EXPERIMENTAL CELL RESEARCH

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FACTS VERSUS ARTEFACTS IN CYTOLOGY: THE GOLGI APPARATUS[1]

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THE word artefact, applied to a cytoplasmic structure, is an effective deterrent to further research, for no one wishes to waste his time in the investigation of a structure whose very existence is in doubt. For this reason it must be considered important to examine critically the recent work of Palade and Claude (21), who have assigned the well known internal reticular apparatus of Golgi to this discouraging category.

The methods used by cytologists in the preparation of cells for microscopic examination are complicated and often brutal. They involve the treatment of the tissue with reagents which either violently precipitate proteins or slowly render them insoluble, followed by a series of solvents both aqueous and organic. The possibility of change is implicit in every step of this process, and it is the prime responsibility of the cytologist to inquire whether the structures which he displays in his preparation actually existed in the living cell, and if not, by what chemical process they have been produced.

It is not surprising that during the history of this science, the results of investigation have been frequently challenged sometimes on theoretical grounds based on biochemical experience, but often as the result of experiment.

The list of structures which have been so challenged is a long one and includes most of the visible structure of the cell. To this list belong, among others, mitochondria, Nissl bodies of nerve cells, myofibrils of smooth and striated muscle, and the chromatin granules of the resting nuclei. All of these have been shown, by subsequent research, to be pre-existent in the living cell.

On the other hand, artefacts have been produced by fixation. To this category belong the network of fibres described by the adherents of the

[1] Study aided by a grant from the Wallace C. Abbott and Clara C. Abbott fund of the University of Chicago.

reticular theory of the structure of protoplasm, and the long filaments produced by Flemming (12) in support of his filar theory. It should, however, be stated at this point that many of Flemming's filar structures were mitochondria which he correctly observed in living connective tissue cells and cartilage cells, and which are well preserved in the peripheral layers of tissue fixed in his chrome-osmic-acetic mixture.

In addition to producing new structures in cells, microscopic technique may and not infrequently does destroy, either by solution or by disintegration, structures originally present. As an example of this, the case of mitochondria may be mentioned. These structures are easily disintegrated by acids, alkalies and organic solvents, which explains why they were so rarely seen by observers prior to 1890. Similarly, secretion antecedents in cells were difficult to preserve prior to the introduction of formaldehyde into technique. In this respect the observations of Richard Altmann (1) stand out as an isolated oasis in a desert of artefacts.

Altmann was much concerned about the possibility of artefact formation and discussed the question at some length but decided, unfortunately, that there was little hope of definitive results from the study of the living cell, because of the small differences of refractive index which were present; a great pity because, with few exceptions, his observations could have been confirmed by direct observation, and his record cleared of the undeserved criticism directed against it as a result of the publication of Fischer's (11) book on Fixierung, Färbung und Bau des Protoplasmas. This work threw doubt on all preceding observations including, and in particular, those of Altmann, and produced a period of nihilism lasting for a decade and a half, during which many investigators were unwilling to concede the possibility even of ultrastructure in a liquid system such as protoplasm.

At this point it may be well to point out that judgments, based on theoretical grounds, of the nature of fixation, rest on rather insecure foundations. Protoplasm is a highly complex system and reactions do not proceed in the crowded molecular medium of the cell as they do in simple systems observed *in vitro*.

What chemist, for example, having observed the precipitation of silver chromate *in vitro*, could foresee that when this reaction was developed in a piece of cerebral cortex, the red-brown silver chromate would be so deposited as to delineate, with exquisite precision, all of the myriad processes of the constituent neurones, or, on the other hand, the beautiful internal reticular apparatus of Golgi. Obviously other factors are present besides the chemical properties of the reacting compounds.

Another factor which has been a source of great confusion is the ubiquitous phospholipin, a constant constituent of protoplasm in not insignificant amounts. The hope of localizing the lecithin and related compounds in protoplasm has been ever apparent in the work of cytologists. For example, Mayer, Rathery, and Schaeffer (18), after an impressive series of analyses of liver in various functional states, and correlation of the results with histological studies, concluded that mitochondria were composed of "lipoid," a view that was shared by many cytologists until Bensley and Hoerr, in 1934, actually separated mitochondria and found that they had a high protein content and were of a highly complex nature.

Later studies showed that mitochondria from the guinea pig liver had a total fat content of about 34 per cent of the dry weight of which about 16 per cent was phospholipin. Similarly, submicroscopic particulates prepared from the same organ had an even higher content of total fats and as much as 26 per cent of the dry weight of phospholipin. In mitochondria at least this fat is not segregated but is dispersed through a highly complex molecular mosaic to which de Jong has given the name coacervate.

Both the mitochondria and submicroscopic particulates have a narrow pH stability range and are easily disintegrated, releasing their components by acids, alkalies and organic solvents. The possibility that the released fats might form myelin figures has received little consideration until recently.

The production of tongue-like projections from the surface of free cells or from naked protoplasts has been frequently observed and was the subject of a special study by Küster (17) who used the plasmolytic methods of de Vries. Gicklhorn (15) however, demonstrated clearly that some, at least, of the processes put out by the protoplasts of the plasmolysed epidermal cells of the onion, were of the nature of the myelin figures seen by workers with nerve tissue or the phospholipins. He found that similar laminated, doubly refractive myelin figures could be produced by treatment of isolated tonoplasts prepared by the methods of de Vries. Gicklhorn also suggested that the Golgi apparatus was produced by a similar release of myelin and the subsequent visualization by silver or osmium. In short, the apparatus of Golgi was an artefact produced post mortem by the release of lecithin, etc. and its combination into bizarre figures such as the Golgi apparatus presents.

In the July 1949 number of the Journal of Morphology, Palade and Claude (21) take up the same theme. In a series of ingenious experiments, they confirm for animal tissues the findings of Küster and Gicklhorn for

plant cells. In addition, they find that the fixing fluids commonly employed for the demonstration of the Golgi apparatus during the slow penetration and unequal rates for the various constituents, liberate "myelin" and produce intracellular and extracellular figures. When the myelin figures are produced in the exiguous confines of the cell, the flow of the myelin is resisted by nuclear membrane and cell mebrane and so forms accumulations of "myelin" in the portion of the cell where the Golgi apparatus is usually found. Hence, they conclude that the internal reticular apparatus which Golgi discovered in 1898, and which has been the subject of innumerable researches during half a century, is entirely an artefact produced post mortem by the liberation of myelin and its subsequent visualization.

There can be no doubt in the mind of anyone familiar with the properties of the intracellular depots of "myelin" as to the accuracy of these observations. The premises and conclusions by which they can be related to the Golgi apparatus may, however, be seriously questioned. On the other hand, they emphasize the immediacy of the problem of reality as regards the Golgi apparatus and indeed, any cytoplasmic structure revealed by complicated chemical procedures.

It is this immediacy which is responsible for the great amount of effort devoted in this laboratory under this writer's direction to devising methods by which the authenticity of protoplasmic structures may be determined. These include methods for visualization of cells *in situ*, of which the introduction by Knisely of the quartz rod illumination is the most important, vital staining methods, separation of cell constituents by differential centrifugation and their chemical characterization, various cytochemical methods, and finally the important freezing-drying method elaborated here by Gersh. Let us now see how these methods of study apply to the Golgi apparatus.

Palade and Claude refer to certain reports on the observation of Golgi apparatus in the living cell but find them unconvincing. They have overlooked the most important ones.

In an article on the pancreas (5), this writer described the canals visible in the cells of the islets of Langerhans of the guinea pig and this observation was extended in 1930 by O'Leary (19), who devised a method by which the islets of Langerhans could be studied in the living animal under the highest powers of the microscope with the blood still flowing in the vessels. Anyone who is familiar with the appearance of the Golgi apparatus as displayed by the classical methods will recognize the similarity to the Golgi figures of the clear canals, seen by O'Leary. These observations

were confirmed independently by Beams (4) in the same year. The introduction of the quartz rod illumination makes the repetition of these observations much easier.

Baker (2), in an article published two months after those of Palade and Claude, reports seeing the Golgi elements in the fresh intestinal cells of the mouse with the phase microscope. He says "The Golgi bodies are the most evident objects in the cell."

The record of vital staining is not so clear. It appears that the vital stains, even such apparently innocuous ones as neutral red and methylene blue, are not above suspicion of causing new formations in still living cells. Yet Covell and Scott (10) appear to have actually stained the Golgi apparatus with neutral red in spinal ganglion cells in which they appear as rows of stained granules aligned so as to present a composite picture very closely similar to the classical Golgi preparation of the same cells. On the other hand Bensley (5), O'Leary (19), and Beams (4), all found the clear canals in pancreatic cells unstained by neutral red.

The introduction of the freezing-drying technique first suggested by Altmann but perfected in this laboratory by Gersh greatly extended our means for deciding whether structures were pre-existent or not. In this method tissues are rapidly frozen by immersion in liquid air, liquid nitrogen or, as suggested by Hoerr, in isopentane, cooled to about —180° C. They are then dried in a high vacuum over phosphorus pentoxide at a temperature of —40° C. or lower. Simpson (22) applied this method to the study of the Golgi apparatus and found that this structure was clearly visible in cells of the epididymis, intestinal epithelium, and in spinal ganglion cells. In all of these the apparatus appeared as clear canal-like spaces in the usual location in sections cut free hand and treated with no reagent except the clearing fluid. The spaces, however, were not empty but contained a substance which stained with Weigert's resorcin fuchsin, thus recalling Emil Holmgren's similar success on staining the contents of his "Saftkanälchen."

Simpson found also that in frozen-dried preparations treated with solutions of osmium tetroxide, the contents of the canals did not stain but the reduced osmium was deposited diffusely in the cytoplasm.

It has been shown that, according to the observations of Bensley, O'Leary and Beams, the Golgi apparatus is visible in the living cells of the pancreas as a system of clear canals. According to Holmgren and Simpson, these canals have a substantive content which stains with Weigert's resorcin fuchsin. Since the next stage in this argument deals with the cytochemical

investigation of the apparatus, it is necessary at this point to discuss its chemical nature. Since the discovery by Kopsch (16) that after prolonged immersion in a solution of osmium tetroxide the apparatus was colored black, the impression that this reaction indicated a fatty composition has been widely held despite the warning of the astute Rudolf Heidenhain. The fact that it takes 3–6 days for this reaction to develop should be enough in itself to invalidate this conclusion, but Owens and Bensley (20) showed that the locus of the reaction could be changed from Golgi apparatus to other cell constituents by changing the preparatory treatment or by addition of electrolytes to the osmic solution. As a result of these experiments, the osmium reaction falls into the same category as the silver chromate and reduced silver methods as a highly dispersed suspension colloid whose locus of deposition is determined by electrokinetic properties of the cell components. These methods of demonstration tell us nothing about the chemical nature of the apparatus.

Another property which has been interpreted as indicating a fatty nature is the fact discovered by Beams that the apparatus is displaced centripetally in the ultracentrifuge. This is inconclusive since a watery content not too heavily loaded with solutes would move in the same direction.

In many preparations made by the osmic acid method, the segments of the apparatus show an unstained center and a deeply stained periphery, the so-called Golgi internum and externum of various authors. This appearance could be produced by the gradual increment of precipitate deposited on the margin or to an actual structure. Recently Baker has shown that a zone around the Golgi apparatus stains with fat stains but since these dyes have to be exhibited in an alcoholic solution which Palade and Claude have shown to be particularly effective in mobilizing the dispersed fats of the cytoplasm, one must reserve judgment as to the primary locus of the fats disclosed.

This leaves us with only the resorcin fuchsin reaction of Holmgren and Simpson, which show a non-fatty positive content of the apparatus which cannot be interpreted chemically but which may be related to the reaction recently discovered by Gersh (14).

It is true that it has been claimed that the Golgi apparatus contains ascorbic acid and phosphatase but while these seem to this writer to be highly plausible assumptions, he does not regard the evidence so far adduced as conclusive.

Gersh's observations, however, are on frozen-dried preparations in which nothing is displaced in the cell and from which the lipins have been ex-

tracted with hot chloroform-methanol. In sections of the intestine he obtained a positive Hotchkiss periodic acid-Schiff reaction in the Golgi apparatus of the intestinal epithelial cells. The apparatus in these preparations had the usual form but the strands were much thinner than in the usual osmium and silver preparations owing to the fact that it was an actual chemical reaction occurring in the substance and not a progressively thickening deposit on the surface. This reaction was negative after treatment with acetic acid but was restored by 0.1 N HCl. It was also negative after peptic and tryptic digestion and weakened by treatment with solutions of ammonium or sodium hydroxide. He concludes "at least tentatively" that the stainable component is a carbohydrate protein complex.

As a result of these observations on the living cell and on frozen-dried cells, one is now entitled to assert, contrary to Gicklhorn, Palade and Claude, that the Golgi internal reticular apparatus is a real constituent of the living cell; that it is not an accumulation of myelin but is composed of spaces containing a watery solution of substances stainable with resorcin fuchsin and in certain cells giving a positive cytochemical reaction with the Hotchkiss periodic acid-Schiff reaction.

On the other hand, the reactions discovered by Gicklhorn and by Palade and Claude are authentic. Lipoids may be mobilized from their dispersed states and appear as droplets or strings by treatment with dilute alcohol and other reagents used for fixation. For this reason, the methods commonly used for the demonstration of the Golgi apparatus cannot be wholly trusted. It seems clear that in many instances structures or artefacts have been wrongly interpreted as Golgi apparatus because they blackened with osmium tetroxide or accumulated silver.

From the foregoing survey, it may be concluded (1) that in the cells discussed, the Golgi apparatus is a real structure demonstrable in the living cell. (2) That it consists of vesicles or canals containing a watery solution. (3) That these vacuoles and canals contain specific substances in solution stainable with resorcin fuchsin and giving a positive reaction for polysaccharides with the periodic acid-Schiff reaction. (4) That one may look forward to further positive cytochemical discoveries through the application of the freezing-drying process.

The question as to whether the Golgi apparatus exists in the form of vesicles or continuous canals has been recently raised by Baker and Gatenby. Baker (2) supports the vesicle idea while Gatenby (13) contends that the structure displayed by the classical methods represents the real form of the Golgi apparatus. The question does not appeal to this writer

as of great importance since slight changes in surface tension may readily convert a canal into a row of droplets or the reverse. The observations on the living cell, however, rather favor Gatenby's view, which is also this writer's.

What then is this mysterious cell organ and what does it do? In spite of 50 years of research, it must be confessed that we know very little and most of our hypotheses are pure guesses based on contiguity of Golgi apparatus and other cell structures. It is high time to wipe the slate clean and proceed along the trails blazed by Simpson and Gersh and endeavor to expand our knowledge of the real chemical nature of the contents of the Golgi apparatus by cytochemical methods.

It may not be amiss at this point to recall that this writer (5), in 1910, published a study in which he attempted to reconcile the structure of animal cells and plant cells. He found that when the meristem cells of plants were fixed in solutions which were adapted to display the Golgi apparatus of animal cells as the canals of Holmgren, the plant cells displayed a complex of clear canals similar to that in the animal cells but of much greater extent. As the cells receded from the growing tip, these canals expanded into the great central vacuole of the plant cell. Hence he concluded that the Golgi and canalicular structures of the animal cell were the strict homologue of the vacuole of the plant cell. This theory was adopted and heavily exploited by Guilliermond and his colleagues, but in the confusion created by the discovery of the neutral red bodies by Parat, which were unfortunately designated the vacuome, this theory was overwhelmed by the welter of arguments about the relation of Parat's vacuome to the Golgi apparatus. Gersh's cytochemical work suggests that the original vacuolar theory still has merit, but I would avoid the use of the word "vacuome" because to many minds it suggests the neutral red bodies of Parat which in the pancreatic cells have been shown by Beams (3) not to coincide with the Golgi apparatus. This work of Beams has been fully confirmed in my laboratory by Chang (9); apparently in this case the neutral red granules are purely artefacts resulting from a process of change similar to those described by Palade and Claude.

The nature of the vacuolar content of plant cells varies from plant to plant and expresses in each the specific metabolism of the cell. It may contain, among other substances, organic acids such as citric, malic, and tartaric and their salts in impressive concentration; mono- and polysaccharides; phenolic and polyphenolic compounds; and inorganic salts in each case specific for the plant concerned.

If the vacuolar theory of the Golgi apparatus should prove valid, a similar variety of chemical content should be expected in animal cells.

In the meantime it should be pointed out that the possession of a large vacuole is not the exclusive property of plant cells. In the flagellate Noctiluca, the cytoplasm occupies only a small portion of the space and the rest of the organism is filled with a clear structureless fluid which Bütschli (8) asserts has a high viscosity. The small fresh water ciliate Trachelius has a similar structure. The highly vesiculated protoplasm of the Heliozoa is familiar to every student of Zoology and the ectoderm and entoderm cells of Coelenterates are often highly vacuolated. The chemical examination of the clear fluid in these cells by microchemical methods should indeed be highly rewarding.

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OBSERVATIONS ON THE GOLGI APPARATUS IN LIVING PLASMA CELLS AND IN ERYTHROBLASTS WITH DARK GROUND ILLUMINATION

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As is well-known, the study of the Golgi apparatus has met with great difficulties and given rise to extensive scientific discussion resulting in controversies. The concepts of this cell organ held by different investigators vary very much. By some it is described as a fibrous reticulum, by others as a ring, a hollow sphere, anastomosing canals or a group of vacuoles.

The method most frequently used for making the Golgi apparatus visible is blackening with silver or osmium tetroxide. The possibility of artefact must therefore be taken into account, at least as regards details of structure. It is significant that the most beautiful reproductions of the Golgi apparatus are drawings, not photographs.

Investigation of the Golgi apparatus in the living cell would certainly be useful. In their admirable survey Kirkman and Severinghous (3), however, give an account of the difficulties involved in the study of living cells. The Golgi apparatus is either not at all visible in tissue cultured cells or does not appear with sufficient distinctness. These authors point out that direct transmitted light, dark field illumination and ultraviolet light photography have so far failed to disclose this apparatus with anything approaching the distinctness of a good osmium or silver impregnation.

It seems, however, that the possibilities of the dark ground illumination method have so far not been fully utilized in the study of living cells. Full advantage of this method is taken only when the object of study is very thin, and teasing out an undamaged living cell under the cover-glass presents technical difficulties. In tissue culture, for example, the cells are as a rule too thick.

In this laboratory it has been possible to observe very thin undamaged

living cells, in which the Golgi apparatus was clearly visible. As these observations may contribute to a proper understanding of the structure and nature of the Golgi apparatus they are reported upon in the following.

MATERIAL AND TECHNIQUE

The cells constituting the object of study were obtained on clinical sternal puncture of patients with severe pernicious anaemia. The blood was therefore very dilute, and the drop of blood was easily spread so that the cells became quite thin. The investigation was carried out with dark ground illumination and a Zeiss apochromatic objective 120W, n.A. 0.85 × K15. The source of light was a Leitz Monla lamp. The cells to be observed being sensitive to short waves, an orange filter was used. The work was performed in the dark or in dim light, since details in the dark ground appear more distinctly when the eye is dark-adapted.

THE MICROSCOPICAL STRUCTURE OF THE CYTOPLASM

When the Golgi apparatus in a living cell is observed under optimal conditions it is found that its structure corresponds in the main to that of the cytoplasm of the leucocytes and their precursors in the bone marrow. This writer has previously (8) described the microscopical structure of the cytoplasm and will therefore only give a summary of those views. Like Schultze (6) the present writer found that the cell body of the human leucocyte contains two kinds of granules or, more correctly, droplets. Minute observation disclosed that they are actually liquid droplets of two different kinds. One of them is highly refractive. The droplets behave like small convex lenses. They refract the light from the diaphragm opening of the microscope to a small image, a round spot of light above the droplet. When a pin's head is placed in front of the diaphragm opening a corresponding shadow appears in each spot of light. This is an optical proof of the existence of the pale droplets. These droplets do not stain vitally and are resistant also to the dyes usually employed in cytological staining.

The other kind of droplets have approximately the same refraction as the surrounding hyaloplasm, which seems to explain why they do not refract the light on elevation or lowering of the tube, as Schultze also pointed out. These droplets appear grey or black on examination of the cells in ordinary transmitted light. In many cells they can be vitally stained and in sections they stain with the usual methods.

The two kinds of droplets are intimately mingled and one or several pale droplets are always found in the immediate vicinity of the dark droplets.

When the leucocytes are observed on a warm stage, rapid movements may be seen in the cytoplasm. It will then be noted that the distance between the dark droplets remains fairly constant even under the greatest activity. It seems as if the dark droplets are surrounded by a sheath preventing them from getting too near each other. If a living eosinophil granulocyte is crushed under the cover-glass after being teased out to a very thin layer, it will fall to pieces. The appearance of one fragment may be that outlined in Fig. 1. Here too the dark droplets (C) are seen at a fixed distance from each other, and the outline of the fragment seems also to suggest the presence of a sheath (B) surrounding them. The pale droplets (A) are found in the sheath adjacent to the dark droplets.

The dark droplets never unite directly. On the other hand they are frequently seen to communicate by means of thread-like anastomoses suddenly forming between two adjacent droplets. To begin with the thread is not clearly visible, but it grows rapidly thicker and is then distinctly discernible. Sometimes a dark droplet is seen flowing over into another droplet through one of these thread-like canals. Often the thread-like bridge is broken up again without any intermediate change in the size of the droplets. When a cell is observed on a warm stage the thread-like bridges are found continuously to appear and disappear.

The pale droplets often run into one another so as to form small rods or sausage-shaped structures, which rapidly disintegrate again into single droplets. The dark and the pale droplets move independently of each other. The dark droplets may remain fairly still while the pale droplets rapidly change position. On the other hand it may be found, especially in injured cells, that the pale droplets lie motionless while the dark ones are continuously communicating by means of the thread-like bridges. Often a dark and a pale droplet, however, move together for a long distance within the cell.

Because of their capacity to stain, the dark droplets have been known for a long time and have been regarded as granules. The chondriosome substance, Altmann's granules, the eosinophil, basophil and neutrophil granules in the leucocytes and their precursors are different manifestations of this dark droplet system.

The pale droplets have escaped attention in stained preparations and in living cells in transmitted light. In dark ground illumination several investigators have noted the small, more or less luminous "granules," which are found in most cells in abundance, but they have interpreted them as identical with the stainable droplets. In dark ground illumination a drop-

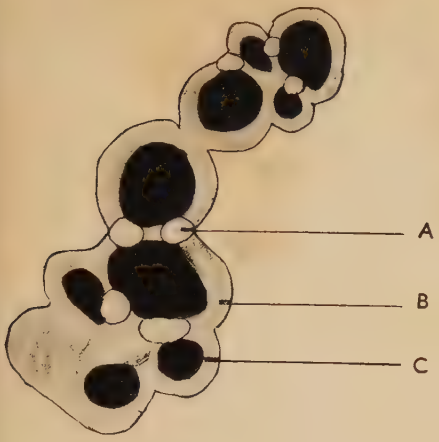

Fig. 1.

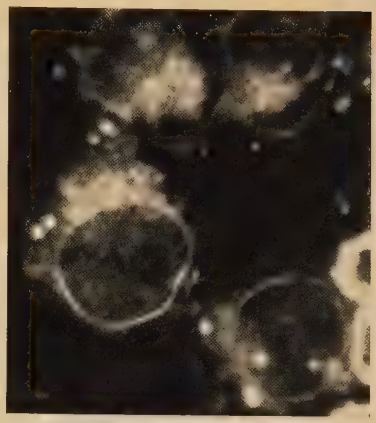

Fig. 2.

Fig. 1. Fragment of cytoplasm from an eosinophil granulocyte crushed while alive. Slightly schematic drawing after photograph. A = pale droplet. B = hyaloplasm. C = dark droplet.

Fig. 2. Living plasma cells in dark ground illumination. The large pale area near the nucleus is the Golgi apparatus.

let is luminous, if it is more refractive than the surroundings, but if its refraction is less than that of the surrounding medium, or if both phases have approximately the same refractive index, it seems optically empty. The dark droplets will therefore seem optically empty in dark ground illumination. As far as this writer is informed, all previous authors have failed to recognize these facts, and therefore also to take notice of the dark droplets. But a worker acquainted with the two droplet systems and used to seeing them in transmitted light will also find the dark droplets in the dark field. They are most easily seen if the cell to be examined is teased out to a very thin layer. Indistinct dark gaps regularly distributed in a faintly luminous ground substance will then be noted in the granulated part of the cell. By observing the movements executed by the dark gaps it is possible to ascertain that they really are manifestations of the dark droplets. Two dark gaps never unite directly, but they communicate by means of temporary thread-like bridges, that is in a manner characteristic of the "dark droplets."

In a previous paper (8) it has been pointed out that the two kinds of droplets described above are not only characteristic of the leucocytes but are found also in other kinds of human cells. The harmonious and regular movements performed by the droplets as well as the exceedingly character-

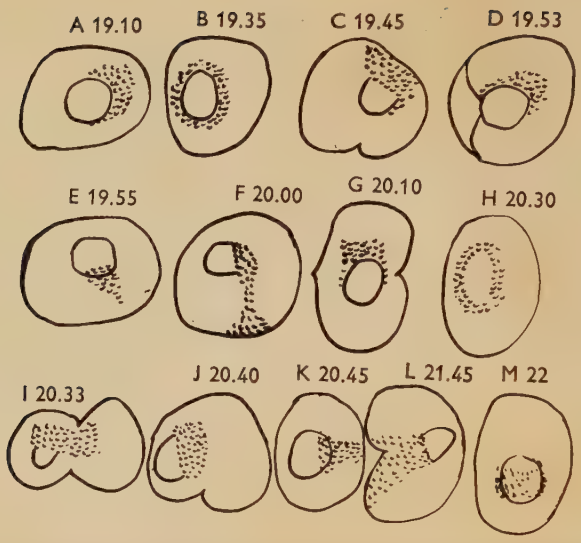

Fig. 3. Drawing of a living normoblast observed at intervals. The numbers above the different drawings indicate the hour at which the drawing was made.

istic interaction of the two droplet systems discernible in the undamaged living cell seem to suggest that the droplets are a very important part of the living protoplasm.

THE MICROSCOPICAL STRUCTURE OF THE GOLGI APPARATUS THE PLASMA CELL

For the purpose of studying the Golgi apparatus *in vivo*, the plasma cell is especially suitable. This cell form is characterized by a pale area near the nucleus, easily discernible in cells that have been variously stained. It is recognized without difficulty in living cells also, in which it appears very distinctly with dark ground illumination (Fig. 2). By impregnating plasma cells with silver according to the methods of Golgi or Cajal, Castrén (2) succeeded in blackening the part of the plasma cell corresponding to the characteristic pale area in the cell body in stained preparations. Thus structures characteristic of the Golgi apparatus were made to appear. The Golgi apparatus coincides, then, with the pale area of the plasma cell. On the dark ground the details of this area appear with special distinctness and may be conveniently studied.

Fig. 2 shows the large Golgi apparatus near the nucleus. Unfortunately the details do not appear as clearly in the picture as they do in the micro-

scope when seen by the dark-adapted eye. The Golgi apparatus has frequently the shape of a crescent and is immediately adjacent to the nucleus. When a living cell is observed on a warm stage, the Golgi apparatus may be seen slowly changing form. It may shift so as to lie over or under the nucleus, and sometimes the nucleus is more or less enclosed by it. In some places it is sharply divided from the surrounding cytoplasm, in others the boundary is less distinct.

It was already mentioned that the structure of the Golgi apparatus is largely the same as that of the rest of the cytoplasm. On the dark field the luminous and the optically empty, dark droplets are seen intimately mingled in a structureless ground substance. The dark droplets are regularly distributed in the ground substance in their characteristic way, and in the Golgi apparatus they shift just as in the cytoplasm. Thus the dark droplets never unite directly, but thread-like passages, or small canals, are frequently formed between two adjacent droplets. These anastomoses are, however, of a temporary nature and suddenly break up again. The pale droplets vary to some extent in size. They move in a characteristic manner between the dark droplets in the structureless ground substance, which is slightly more luminous than the surrounding cytoplasm. From the Golgi apparatus small processes, which gradually change shape, project into the surrounding cytoplasm. The microcentrum, demonstrated by Maximov, Weidenreich and A. Wallgren (4, 7, 10) in the pale area in the stained plasma cell, is found also in the living cell in the form of an ungranulated spot in the Golgi apparatus. Less luminous, pale grey areas showing a structure resembling that of the Golgi apparatus, though less distinctly discernible, are seen here and there in the surrounding cytoplasm. Other parts of the cell body are optically empty.

As already mentioned, the boundary between the Golgi apparatus and the rest of the cytoplasm is not quite sharp. Communication between the Golgi apparatus and its surroundings is actually seen to take place in the living cell. On the dark ground luminous droplets are seen to leave the Golgi apparatus and move about in the cytoplasm over a long distance. Once a droplet left the Golgi apparatus, described a curve in the cytoplasm and again found its way into the apparatus.

THE MEGALOBLAST

In living megaloblasts from persons with untreated pernicious anaemia the Golgi apparatus is seen as a corona-like sheath around the nucleus.

The boundary dividing it from the rest of the cell body, which is optically empty, is irregular inasmuch as corners of the sheath-like corona protrude into the surroundings. In the living cell they gradually change in shape and appearance. The structure of the Golgi apparatus is identical with that of the plasma cell. Here too we find a similar double droplet system with dark and luminous droplets in a surrounding, faintly luminous ground substance. Perhaps further mention should be made of the fact that hematologists are not familiar with the Golgi apparatus in the megaloblast but have noted a pale uncoloured ring-shaped zone surrounding the nucleus in the otherwise dark cell body.

THE NORMOBLAST

The cell body of the normal living nucleated human erythrocyte is optically empty, but near the nucleus a luminous area is seen on the dark field, exhibiting a structure resembling that of the cytoplasm of leucocytes. There can scarcely be any doubt as to its being the Golgi apparatus of the cell. This writer has previously (9) reported his observations on the disappearance of the nucleus from the normoblast, and these findings were corroborated by G. F. Saltzman (5). Our investigations proved that the Golgi apparatus during the process of maturation continuously changed shape and performed movements in the optically empty cell body. In Fig. 3 drawings are reproduced of a living normoblast, observed at intervals. The numbers above the cell indicate the hour at which each drawing was made. As appears from the figure, the cell body gradually changed shape and the position of the nucleus within the cell body was somewhat shifted. The changes in shape of the Golgi apparatus appear clearly from the figure. It will be noted that contact with the nucleus is never interrupted. The nucleus is always more or less, and in Fig. M entirely, surrounded by the Golgi apparatus. The changes in shape of the apparatus take place rather rapidly. In the space of a few minutes the Golgi apparatus has changed place and shape. (Compare for instance Fig. B and C, or F and G.) Occasionally the changes are so rapid as to be directly discernible. During one phase of the maturation process the nucleus is pushed out of the cell so that it lies bare and is retained by the cell body only by means of the Golgi apparatus. Later on the nucleus shrinks, is enclosed by the Golgi apparatus and dissolves.

Minute examination shows that the structure of the Golgi apparatus of the normoblast is identical with that of the plasma cell. In this case too

the dark ground microscope reveals that dark and refractive droplets are arranged in the characteristic manner previously referred to, in a faintly luminous ground substance. The dark droplet system is vitally stainable, staining for instance with brilliant cresyl blue. The thread-like passages between the dark droplets often appear very distinctly. Especially continuous forming and disappearing again near the shrunken nucleus have been observed. When the nucleus is dissolved, the Golgi apparatus remains. The dark droplets and the thread-like bridges between them are then easily seen in preparations stained with brilliant cresyl blue, and are well known to hematologists under the name of substantia reticulofilamentosa. The dark and the pale droplets having been ejected, the reticulocyte is changed into an erythrocyte.

DISCUSSION

The investigations described above have shown that, in the various cell forms, the Golgi apparatus is always in immediate contact with the nucleus. This is most distinctly seen in the normoblast, in which the Golgi apparatus is always more or less intimately united with the nucleus in spite of considerable movement within the cell body. In all of the cell forms examined it was noted that the Golgi apparatus is capable of changing shape, but in this respect also the tendency was most marked in the case of the normoblast. With dark ground illumination the finest structures in the Golgi apparatus are invariably the same: A faintly luminous ground substance, in which the optically empty "dark droplets" lie evenly distributed at fairly regular distances from each other, surrounded by the small luminous pale droplets. Between the dark droplets thread-like anastomoses are sometimes observed. It thus appears that small liquid-conducting canals may be present in the Golgi apparatus. In the living cell they are of a temporary nature, but in an injured or fixed cell they can be preserved even after the death of the cell.

The structureless ground substance surrounding the dark and the pale droplets in the Golgi apparatus is slightly more luminous on the dark ground than the rest of the cell body, which seems to indicate a somewhat higher refractive index. The movements performed by the ground substance are, however, identical with those observed in the hyaloplasm of the rest of the cell body.

The relationship between the chondriosome substance and the Golgi apparatus is particularly interesting. In the lymphocyte the chondriosome

substance is clearly localizable to the hilar region near the kidney-shaped nucleus. In the living cell the dark droplets stain deeply with Janus green in that region, while the pale droplets remain unstained. As is well known, vital staining with Janus green is one characteristic of the chondriosomes. It is thus obvious that the dark droplets near the nucleus of the lymphocyte contain chondriosome substance. When the two droplet systems are gathered near the kidney-shaped nucleus, this part of the cell, which is luminous on the dark ground, very much resembles the Golgi apparatus of the plasma cell, and it may very likely be the Golgi apparatus of the lymphocyte. Both in the plasma cell and in the normoblast the dark droplets of the Golgi apparatus also stain with brilliant cresyl blue.

On staining cells for chondriosomes, these often appear visible near the nucleus in the same region where the Golgi apparatus turns black during impregnation. If the dark droplets in the living Golgi apparatus contain chondriosome substance a natural explanation of this circumstance is afforded. It was pointed out already that an exchange of droplets was seen to take place between the Golgi apparatus and the rest of the cytoplasm in the living plasma cell.

Just as in the case of the cytoplasm, the Golgi apparatus may also be compared to a three-phase system, where two of the phases, i.e. the pale and the dark droplets (the chondriosomes), are dispersed in a continuous phase ("the Golgi material"). It is therefore not surprising that the Golgi substance and the chondriosome substance are separated in ultra-centrifuged material by several authors, see (1).

For the purpose of attaining a clear concept of the microscopical structure of the Golgi apparatus, the study of a living cell, well teased out and properly illuminated on the dark ground is recommended. In a living cell the droplet systems of the Golgi apparatus perform rapid movements, and thus not only the distribution of the two kinds of droplets in a structureless ground substance appear, but the characteristic movements of the different phases will also help to elucidate the structure of the Golgi apparatus. If the observations on living cells are compared with blackened, fixed cells, the details of the silver- or osmium-treated Golgi apparatus will be identified. In the fixed cell, the ground substance of the Golgi apparatus, which surrounds the pale and the dark droplet systems, will form a three-dimensional network composed of strands and previously described as the "Golgi material." It will be found that the outer boundary layer of the apparatus or the strands have turned black, or that the silver or the osmium tetroxide have been segregated in the layer nearest to the dark droplets. Occasionally

a rich aggregation of thread-like bridges, fixed so as to form reticular structures, is found between the dark droplets.

It is also obvious that the microscopical structure of the Golgi apparatus will not be clearly understood from examination of blackened preparations alone. The mystery in which Golgi's apparatus is still wrapped can only be dissolved by observation of the living cell, and by this method only will it be possible to explain its complicated structures.

SUMMARY

The living Golgi apparatus has been studied in very thin preparations with dark ground illumination. Two kinds of intimately mingled droplets are found in a structureless ground substance. The movements performed by the two droplet systems are described in detail. The Golgi apparatus is capable of changing shape and is mobile. An exchange of droplets was observable between the Golgi apparatus and the surrounding cytoplasm.

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ESTIMATION OF PHOSPHATASE IN HISTOLOGICAL PREPARATIONS

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Since 1939 several hundred papers have been published dealing with the histological localization of phosphatases. Most of these employ methods primarily developed by Gomori (8, 9) which depend upon precipitation in the section of one moiety of the hydrolysed substrate, e.g. the phosphate of glycerophosphate, by means of an ion such as calcium or lead. This precipitate is then transformed into the more readily visible cobalt or lead sulfide. When due cognizance is taken of preformed substances yielding sulfides, the sites of phosphatase activity are considered to be selectively blackened by this »indirect» histochemical test.

That the procedures give positive reactions in tissues containing phosphatases has been repeatedly demonstrated (1, 13). Whether the sites of the sulfide deposition represent the true loci of enzyme activity has been discussed in various critical studies (2, 10, 12). Several attempts have been made to interpret the staining reaction in semi-quantitative terms by visual assessment or direct photometry, but the validity of these methods is questionable (11). The visual microscopic appearance of sections has been related to independent direct chemical assay for substrate hydrolysis in comparable material (2, 5). The "direct" assay and the "indirect" sulfide estimation have many differences of procedure although some of the histological preparatory steps can be incorporated prior to "direct" assay (3, 15), and their effects evaluated. It is assumed in "direct" assay for phosphatase that the investigator has shown that the reaction product measured has been produced in a known relationship to enzyme content and activity, and that suitable "blanks" or "controls" for non-enzymatic reactions have been incorporated in the procedures. In the histological literature on "in-

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direct" histochemical preparations there has been a notable lack of attention paid to parameters other than the use of "blanks" of various sorts and varied incubation times. By a modification of a procedure for the estimation of H_2S as methylene blue (7), it is possible to assess the amount of sulfide in histological preparations (4). A quantitative measure of the total sulfide precipitated in a section permits the description for "indirect" phosphatase estimations of some of the parameters which are required to demonstrate the extent to which the procedure is truly quantitative. In addition it makes possible a measurement of some of the effects of the peculiarly histological steps involved.

The present study is concerned with the relation between the amount of enzyme, time of incubation and the amount of sulfide deposited following the "standard" procedure which is described. The effects of some relatively minor differences in procedure, such as might exist between two laboratories working on the same material are noted and some inferences as to the state of alkaline phosphatase in kidney sections are drawn. Conditions for obtaining quantitative conversion of sulfide to methylene blue in sections on slides are described.

MATERIAL

The enzyme content of individual preparations was regulated by varying the area and thickness of the sections. A tissue from which several nearly identical sections could be cut was required, and tangential sections of the rabbit kidney cortex were found to be suitably homogeneous. A few experiments on rabbit appendix and rat duodenum are included.

METHODS

a. Estimation of Sulfide.

In the method of Fogo and Popowski (7), H_2S is trapped as zinc sulfide. The zinc sulfide is decomposed with acid in the presence of para amino dimethyl aniline sulfate (E. K. Co), (PADMAS) and upon the addition of ferric chloride, methylene blue is formed. The usual histochemical procedure for alkaline phosphatase results in the precipitation of cobalt sulfide which is not sufficiently soluble in hydrochloric acid to be readily estimated as methylene blue. By substituting lead for cobalt, a sulfide is obtained which decomposes rapidly in 5N HCl. In the modification reported by Doyle (4) the sections were placed at the bottom of small 6×25 mm test tubes and liquid seals employed to prevent the loss of H_2S. The ferric chloride was introduced through the seal (Fig. 1 d). The test tube method is more precisely quantitative than the new one for the estimation of the sulfide in a single given sample, but it requires

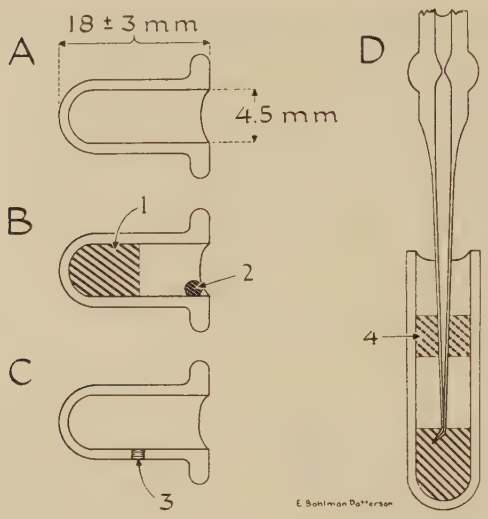

Fig. 1. Reaction vessels for sulfide determinations. In use the glass surfaces are coated with hydrophobic silicone. A, B, C vessels used for small sections on slides. B, (1) position of Padmas solution B, (2) position of acid. C. Vessel used to introduce ferric chloride through the 2 mm hole (3). D. Arrangement used for introducing ferric chloride through a liquid seal (4) when sulfide estimations are done in micro tubes.

that each section be passed separately through the Gomori procedure. Since this involves fifteen steps, some of which must be precisely timed, it is more difficult to insure that all of a set of twelve or more sections under comparison have been treated identically when handled individually. The section in the test tube is also less readily examined microscopically than one on a slide. Accordingly, a system for treatment of sections on slides was devised.

Consecutive tissue sections were mounted, two to a slide, and processed as described below for acid or alkaline phosphatase. One section was made into a permanent mount for microscopic examination while the other was allowed to dry.

To avoid loss of H_2S during the reaction special tubes were made (figures 1 and 2) of various sizes to accomodate a wide range of sulfide values. These tubes have a flat flange at the mouth which may be smeared with silicone stop-cock grease to afford a seal to the slide when clamped in position. The tubes are coated with G. E. Dri-film No. 9987[1] to provide a hydrophobic surface in order that two solutions may be manipulated separately and successively in the closed system. The larger tubes require a constriction (neck) to permit this manipulation.

Reagents are added to the tubes in the following proportions their volumes being dependent on the size of the tubes.

[1] The hydrophobic coating was applied as a 5 per cent solution of Dri-film in chloroform which was then drained off. The tubes were then dried at 110° C for a few hours, and then boiled in several changes of distilled water to remove excess acid, and finally dried at 110° C.

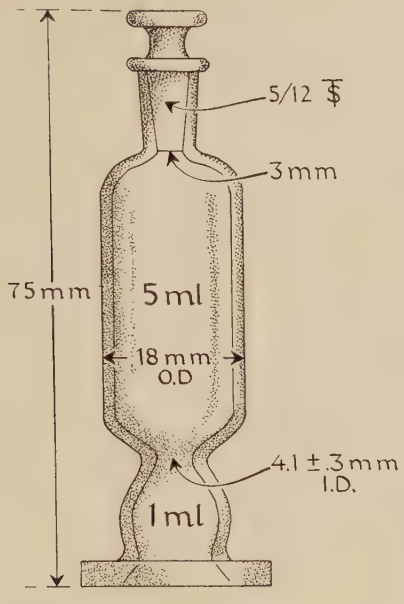

Fig. 2. Vessel used for estimation of sulfide in sections up to 8 mm in diameter or containing up to 4 μg of sulfide. The upper, 5 ml, portion contains the PADMAS solution which owing to the hydrophobic coating can be retained above the neck if the constriction is not larger than 4.3 mm. When the vessel is held horizontally the 1 ml section contains the acid. A wide flange is provided at the base to facilitate clamping to the slide. Ferric chloride is introduced through the stopper.

1. To the bottom of the tube add 100 ±2 μl of a solution "A" of para amino dimethyl aniline sulfate (PADMAS) prepared by mixing 10 ml of stock solution with 49 ml of water. The stock solution contains 0.100 gm of PADMAS in 100 ml of 2.7 N HCl and is stored in the ice-box.
2. The flange at the mouth of the tube is smeared evenly with silicone stop-cock grease.
3. Near the mouth of the tube, while it is held horizontally, add 8.5 ±.2 μl of 5.4 N HCl.
4. With the tube held horizontally, apply the slide to the mouth of the tube so as to enclose the section.
5. Invert the tube by turning the slide into a horizontal position. Tap or shake the slide gently so that the section is covered with acid without bringing down the PADMAS solution. When the section is bleached by solution of the sulfide, clamp the tube and the slide together.
6. Shake the tube vigorously to mix the solutions thoroughly and to aid in bringing the H_2S into solution. Remove the clamp. This step should be completed in a constant time interval, preferably less than three minutes.

7. By means of the small hole (Fig. 1) or stopper (Fig. 2) or by sliding the tube to the edge of the slide to open a narrow slit, introduce 3.4 ±.1 μl of 0.023 M ferric chloride in 1.2 N HCl. Close vessel immediately and shake thoroughly.
8. Allow color to develop 20–30 minutes at 25° C and read in the spectrophotometer at 670 mμ.

   For maximum precision the temperature of all reagents should be 24 ±1 C°. The color of the methylene blue solution in the tubes (before dilution) should not exceed an optical density of 0.8 for a depth of 1 cm at 670 mμ to ensure the complete reaction of H_2S. The volume of liquid phase in the closed system should be at least 65–90 per cent of the total volume of the vessel (especially at the higher concentrations).

Preparation of a standard curve for sulfide estimation may be carried out as described by Fogo and Popowski (7) or as follows.

Into a 125 ml glass-stoppered iodine flask introduce 100 ml of PADMAS solution A. With a micro-balance weigh out 0.05–0.6 mg samples of finely divided lead sulfide in short test tubes. Introduce the test tube into the flask and add 8.5 ml of 5.4 N HCl to the sulfide in the test tube and stopper immediately. When the sulfide is dissolved (which may require several minutes), shake the flask well. Add 3.4 ml of ferric chloride solution to the well above the stopper and then lift the stopper to introduce the ferric chloride solution with a minimum escape of gas. Shake well and allow the color to develop at 25° C.

b. "Direct" Assay of Phosphatase Activity.

Acetone-fixed paraffin sections were placed in Carlsberg test tubes (6 × 25 mm outside dimensions) and deparaffinized with three changes of benzene which was removed by aspirating with a fine pipette. The sections were then rinsed with acetone which was aspirated off and allowed to evaporate in a stream of air. To the section at the bottom of the tube was added 15 μl of N/10 ammonium hydroxide-ammonium chloride buffer, pH 9.8, and it was allowed to stand for 20 minutes. To the buffer an equal volume of 0.5 per cent disodium phenylphosphate was added and the tubes were incubated at 40° C for 30 minutes. The reaction was stopped by addition of carbonate and Folin-Ciocalteau phenol reagent and the color allowed to develop for 30 minutes. The color was read in a Beckman spectrophotometer using Lowry micro cuvettes. Controls on the sections and substrate were run and standards containing 0.5, 1.0 and 2.0 μg of phenol were included in each series. Acid phosphatase activity was similarly determined at pH 5.4 using 0.1 M acetate buffer.

c. Histological Methods.

In the basic procedure, pieces of tissue not over 3 mm thick, were dropped into 50 to 100 volumes of acetone at 0 to —5° C and the bottle transferred to the deep freeze at —20° to —25° C for 24 hours. The acetone was then replaced with fresh acetone at the same temperature and stored at —20° C until embedded in paraffin. In the embedding process the tissues were brought to room temperature and the acetone replaced with benzene. When cleared the tissues were placed in molten paraffin at 58°–60° C for 15 minutes, then transferred to paraffin in vacuo at 60° C

for 20 minutes, and finally blocked in a third change of paraffin. Blocks were stored in a refrigerator at 5–8° C until sectioned. Sections were mounted dry with pressure on albumen coated slides and allowed to stand one hour at room temperature or longer at —20° C. They were then deparaffinized in two changes of benzene, rinsed in acetone, and transferred to 80 per cent alcohol for 10 minutes to harden the tissue. From 80 per cent alcohol they were rinsed for 5 seconds in water and placed in the substrate at 30° C for the required incubation time. Variants of this procedure are described in connection with specific results.

In order to compare sections of different thicknesses a group of six sections was cut at a stated thickness, the microtome was then reset and six more sections cut at the new thickness and so on. The first two sections in each group were discarded to avoid possibility of unequal thickness.

Slides were processed according to the following schedule.

1. Incubation in substrate for the time stated at 30° C.

Substrate solution	pH 9.3–9.5
2 per cent sodium a, b, glycerophosphate	100 ml
2 per cent sodium barbital	100 ml
Distilled water	200 ml
2 per cent calcium chloride	20 ml
2 per cent magnesium sulfate	8 ml

The glycerophosphate solution and the substrate mixture were stored in the refrigerator and were made up fresh weekly or oftener. The substrate solution in the Coplin jar was used only once.

2. Rinse in water 5 seconds.
3. Three changes of distilled water at 2 minute intervals.
4. Treat with 0.1 M $PbNO_3$ for 10 minutes except for sections with very high activity which are placed in 0.2 M $PbNO_3$ for 20 minutes.
5. Repeat steps 2 and 3.
6. Ammonium sulfide—strong solution diluted 1:50 — for 10 minutes.
7. Rinse thoroughly in water.
8. Dry in air for sulfide determination or dehydrate with alcohol and make a permanent mount.

Results with Standard Procedure

1. Relation between amount of tissue and amount of sulfide deposited.
   a. Variation in Area. A small block of tissue about 2 sq mm in area was sectioned and the sections were mounted so that 1, 2, or 3 sections could be analysed in a single determination. The amount of sulfide per section was found to be constant $\pm$ 2 per cent thus affording a check on the accuracy of the method.
   b. Variation in Thickness. Several blocks of kidney tissue (cortex) were cut so as to give groups of sections at 3, 6, 9, 12, and 18 μ or 4, 8,

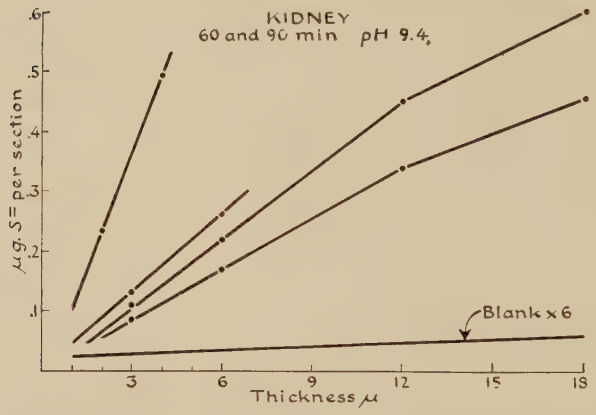

Fig. 3. Relation between section thickness and sulfide formation in four samples of kidney cortex. The three central curves extrapolate to the origin.

and 12 μ. Typical results are shown in the graph Fig. 3, from which it can be seen that for incubation periods of 60–90 minutes the amount of sulfide deposited is proportional to the thickness of the section up to at least 12 μ and in most instances there is approximate proportionality up to 18 μ thickness.

Discussion — Standard Procedure

1. Relation between tissue area and thickness and the amount of sulfide deposited.

The linear relationship found between the amount of tissue and sulfide deposit in sections incubated for 60–90 minutes (Fig. 3) indicates that the reactions of the standard procedures are not limited by diffusion processes in sections 3–12 μ thick, but that there is some limitation in some 18 μ sections. If the enzyme or the various precipitates are lost from the sections by diffusion into the media, the loss must be proportional, regardless of thickness or the lines in the graph would not go through the origin. A few cases with sections thinner than 3 μ indicate a lowered activity in the thinner sections, but the opportunities for accidental loss of tissue are greatly increased with the thin sections.

In preliminary experiments it was sometimes found that a straight line relation between thickness of section and sulfide deposition occurred, but that the extrapolated value for zero thickness was greater than zero. Such a relationship would

exist, if, with increasing amounts of enzymatic reaction product there was: 1. less complete conversion of calcium phosphate to lead phosphate; 2. less complete conversion of lead phosphate to lead sulfide; 3. increased loss of hydrogen sulfide in the methylene blue formation. Other factors which might operate to produce this result are: 4. the rate of diffusion of substrate into the section may limit the reaction. This would be expected with substrates of low diffusibility and with thick sections, and possibly with coated sections; 5. the desposition of calcium phosphate around the site of enzyme activity may progressively inhibit access of the substrate to the enzyme, especially at long incubation periods or high enzyme activities.

In this work such results were eliminated by correcting point 3 by means of improved techniques, but the other possibilities are listed here because they have been frequently raised in discussions. Under the conditions finally used in the work reported here, the remaining four points are excluded in the experiments in which the lines go through (or below) the origin. We have only rarely obtained results in which the lines go below the origin when using the standard histological procedures.

In discussions of false localization of sulfide, a factor frequently raised is that of diffusion of one of the incompletely insoluble reactants. If diffusion into the substrate solution occurs one might expect a greater proportionate loss from thin sections than from thick ones. This situation would result in increased slopes in graph figure 3, and should produce intercepts at negative values. That such negative intercepts have not been observed may be due to the predominant effects of the factors previously listed as contributing to positive intercepts. (But see results with variations in the histological procedures.) In view of the variables employed, however, it would be remarkable if the opposing factors were so precisely balanced as to give the large proportion of zero intercepts that was observed.

2. Relation between incubation time and sulfide deposition.

With sections of 3–12 μ thickness incubated for periods of 1 $^1/_2$ to 2 hours, the sulfide deposit is proportional to the time of incubation after an initial lag period (Fig. 4). In the standard procedure slides were transferred from alcohol to substrate with a very short rinse in water. When the procedure was modified to provide a period of hydration in control medium (substrate mixture lacking glycerophosphate) prior to transfer to substrate, the lag disappeared. 30 to 60 minute incubations of 10 μ sections show a maximal effect of prehydration for 10 minutes; there is no significant difference for a two hour incubation period, and only 5 per cent at 60 minutes. For all the sections used, a prehydration period of 20 minutes was adequate and there was no loss of alkaline phosphatase before the sections were placed in the substrate. There is some evidence that sections thinner

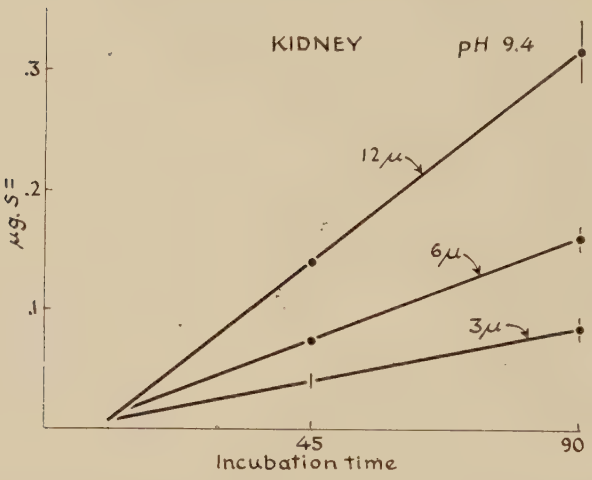

Fig. 4. Relation between incubation time and sulfide formation when deparaffinized sections of kidney cortex are placed in substrate without prior hydration. The vertical lines indicate the range of variation of the determinations.

than 8 μ are hydrated in about 5 minutes, whereas sections thicker than 10 μ require 10 minutes.

When incubation periods of $1\,^1/_2$ to 6 hours are used, the rate of sulfide precipitation may show a change after 2 or 4 hours to a lower fairly constant rate, Fig. 5. The decrease occurs earlier at higher pH values and varies markedly with the tissue. The change in rate may be attributed to 1) inactivation of the enzyme with time, 2) occlusion of the enzyme by precipitated calcium phosphate, or 3) formation of a calcium phosphate deposit which is very dense (or so chemically changed) so that it is less quantitatively converted to sulfide. The latter may be related to the apparently decreased precipitation of silver noted by others (14). The constancy of the lowered rate might occur by occlusion of the most active sites of enzyme activity, leaving the less active areas still functioning. In our experiments the decreased rate could be observed when the amount of sulfide exceeded 0.1 μg per sq. mm and in such cases there were some deposits at sites of maximal activity which appeared to have black surfaces and lighter interiors.

As set forth above, the sulfide content can be a consistent index of enzyme activity. These experiments show that within certain limits and with allowance for non-enzymatic reactions the amount of sulfide deposit (but not necessarily its visible blackness) is directly proportional to the active

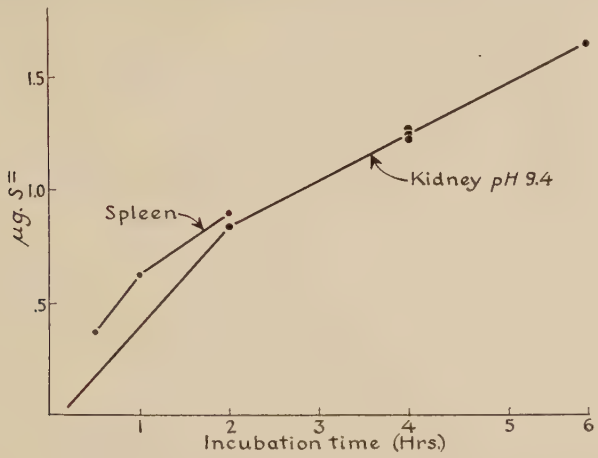

Fig. 5. Relation between sulfide deposition and time of incubation for longer periods of incubation. A decreased rate is observed after 1 hour in the case of spleen (pH 9.4) and after two hours in kidney cortex sections.

enzyme content of the section. It is, of course, easier to measure the phosphate precipitated in the incubated sections by direct assay (Fiske and Subbarow) rather than by conversion to sulfide and subsequent estimation of methylene blue.

To check the relation between the calcium phosphate precipitated and the sulphide formed, an experiment was set up involving a fairly lengthy series of consecutive sections. Holter and Krugelis (personal communication) had noted that the cobalt sulfide precipitate of the usual procedure was admixed with phosphate. Accordingly the original calcium phosphate, the sulfide, and the phosphate left after conversion to sulfide were measured in consecutive sections in groups which had been incubated in substrate for $^1/_2$, 1, $1\,^1/_2$, 2, 3, 4, and 6 hours. The phosphate was measured using the vessels described for the sulfide determinations; the sections were treated with acid, and if sulfide was present the H_2S was removed by aeration, and the Fiske and Subbarow reagents added.

Since sixty 15 μ consecutive sections were used it is necessary to take into account some of the histological variations in the sections. The kidney cortex from a two month old male rabbit was fixed in chilled acetone for 24 hours, transferred to cold absolute alcohol for 24 hours and then embedded in paraffin. Sections 15 μ thick and 10 sq. mm in area were obtained. The outermost sections contained fewer and smaller (younger)

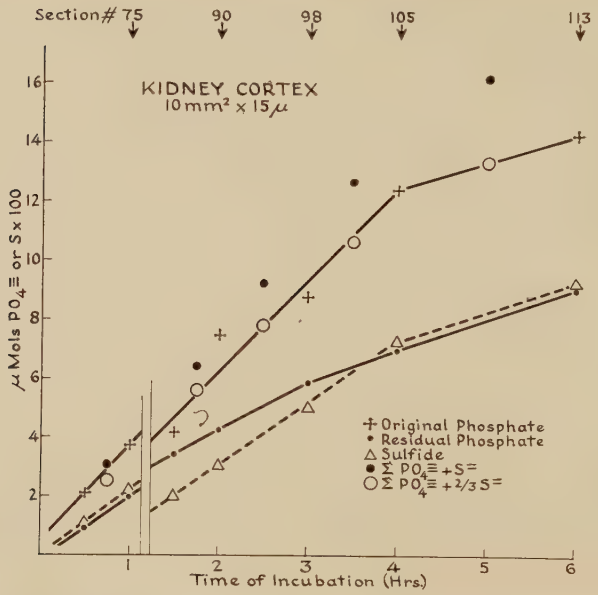

Fig. 6. Relation between time of incubation and precipitate formation in kidney cortex sections incubated at pH 9.4 in substrate solutions. The upper curve is drawn to the best fit for original phosphate values. The large circles (○) fit this line better than the solid circles (●).

glomeruli than sections deeper in the cortex. Since the glomeruli show an amount of alkaline phosphatase which is negligible in comparison with that in the proximal tubules, there is accordingly a decrease in the effective area of the section. The changes in composition of the sections are most abrupt between sections No. 75 and 80 (the 60 and 90 minute incubation groups) and this is indicated in figure 6 and in the Table I. The actual total area of glomeruli accounts in very small part for the break in the curves between sections 70 and 83. This feature is thus only an index of histological inhomogeneity and other differences such as proportions of proximal tubules etc. are presumably involved. Within each group of 8 sections at a given time of incubation, however, these changes may be considered to be negligible.

The curves (Fig. 6) show that about 60 per cent of the original phosphate is present after treatment with lead nitrate and ammonium sulfide, with little effect of varied time of incubation. On a mol basis the sulfide formed approximates the residual phosphate. There are all too many possible reactions which might be involved between the original precipitation of

TABLE I

Distribution of Glomeruli.

Section No.	No. of Glomeruli	Av. Radius of Glomeruli (mm)	Area of Glomeruli × No.[1]	Per cent of Section Area
69–70	49	.039	.235	2.5
83–84	70	.047	.485	5
85–86	73	.049	.55	5.5
93–94	73	.048	.53	5
101–102	74	.047	.51	5
109–110	67	.047	.45	4.5

calcium phosphate (from an alkaline medium), its treatment first with lead nitrate (at pH 4.4) and subsequently with ammonium sulfide. Precipitated calcium phosphates have compositions which depend upon pH and which can exchange or take up other ions. Lead salts are particularly prone to complex formation. If we assume that the sulfide represents bivalent lead which was combined with trivalent phosphate we may sum the values for residual phosphate plus two-thirds the sulfide values to get the points marked (0) on the graph. Such values coincide very well with the values for the original phosphate (+). At the shorter incubation times the sums of residual phosphate and three-thirds the sulfide match the original phosphate values about as well, but at longer periods the deviation is marked.

Although the conversion of calcium phosphate to lead sulfide is far from complete, there appears to be a roughly stochiometric proportionality which under standardized conditions validates the use of sulfide content as an index of phosphate precipitation. That the explanation is not as simple as this is shown by experiments involving the floating of these sections on water when they were mounted on slides. Although the sections were hardened in alcohol before embedding they do show a slight increase in total phosphate precipitated as a result of stretching the section (cf. results of variant procedures, below). In floated sections the residual phosphate is lower, and the sulfide higher, than in comparable dry-mounted sections. Thus the physical dispersal of the reaction sites favors an increase in proportion of phosphate converted to sulfide.

[1] = Total area of glomeruli per section (sq. mm).

Results of Varying the Histological Procedure

1. Effects of floating the sections on water when mounting. Sections prepared by the standard histological procedure (p. 24) were compared with respect to total sulfide deposition with sections treated as follows:

   A. Dry mounted but without hardening in 80 per cent alcohol.
   B. Mounted by floating the acetone-fixed, paraffin sections on water.
   C. As for B followed by hardening in 80 per cent alcohol.

Method A leads to the swelling of the sections in the substrate, the amount varying greatly with the tissue used. This is eliminated in the standard procedure by hardening in 80 per cent alcohol before introduction of sections into aqueous solutions. Methods B and C result in marked distortion of lymphatic tissue but kidney sections are less damaged.

After processing such preparations, method C shows maximal deposition of sulfide; the standard method is next; the dry mounted, unhardened section (A) next; while the floated, unhardened section (B) gives the least sulfide deposit. The B and C preparations were "cleaner" in the sense of having an almost colorless background whereas hardened preparations were faintly yellow. The outer border of floated sections showed a faintly staining material, apparently translocated by the floating process. If one assumes that unhardened sections lose enzyme during incubation, the low values for methods A and B are explicable. The maximal reaction with method C was unexpected.

In accounting for these results it is necessary to consider the findings with similar treatment of sections which were assayed by the "direct" determination of phosphatase activity. It is important to note that in this procedure, unlike that of the sulfide determination, any enzyme carried into the substrate medium is measured as well as that left behind in the section.

The standard "direct" procedure employed sections which were acetone fixed but not hardened in alcohol. To obtain variants of technique corresponding to the flotation and hardening of sections on slides the sections in test tubes were extracted with the same volume of water (10 μl) as used for floating sections on slides and they were hardened by immersion in alcohol.

The results of the direct assay after such treatment were: the unhardened sections were the most active (standard "direct" procedure), the unhardened sections which were extracted for 5 minutes with water gave essentially the same values, but control values for preformed phenolic substances were

TABLE II

Comparison of effects of floating sections on microscopic and direct phosphatase estimations.

Treatment of Kidney Cortex sections (10 μ)	Relative activity (arbitrary units)	
	Sulfide determination in the section	Direct Phosphatase determination
Acetone fixed then transferred to substrate	5.0	10.0
Acetone fixed then extracted or floated on water	4.0	9.8
Acetone fixed, alcohol hardened	8.0	5.0
Acetone fixed, extracted, then alcohol hardened	10.0	7.5

reduced. The alcohol treated sections had about 50 per cent of the activity of the unhardened sections, but sections which were extracted in water and air-dried prior to hardening in alcohol had 70-80 per cent of the values for unhardened sections. The "extraction" prior to hardening in alcohol lowered the control values and resulted in preparations with about 50 per cent more activity than those hardened without water treatment. These findings are tabulated (Table II).

The above values were obtained with kidney sections 10 μ thick incubated for one hour (sulfide) or for 30 minutes (direct) under conditions in which no inactivation of the enzyme occurs during incubation.

The greatest disparity between the results of the two methods lies in the fact that from the sulfide deposition one would not suspect the inactivation by alcohol which is observed in the direct determinations. In fact, the unhardened sections show lower sulfide deposition. On the assumption that unhardened sections lose enzyme to the substrate, the two sets of results can be reconciled since the direct method measures the extracted enzyme as well as that left in the section. It is true that the 5 minute "extraction" period, with water, results in negligible loss of enzyme in direct determinations but such sections still contain paraffin and some of this time is required to rehydrate the section.

The results of $S^{=}$ determinations for sections of various thicknesses, figure 7, shows a steeper slope for floated sections than for those mounted dry. The dry-mounted sections give a line which passes approximately through

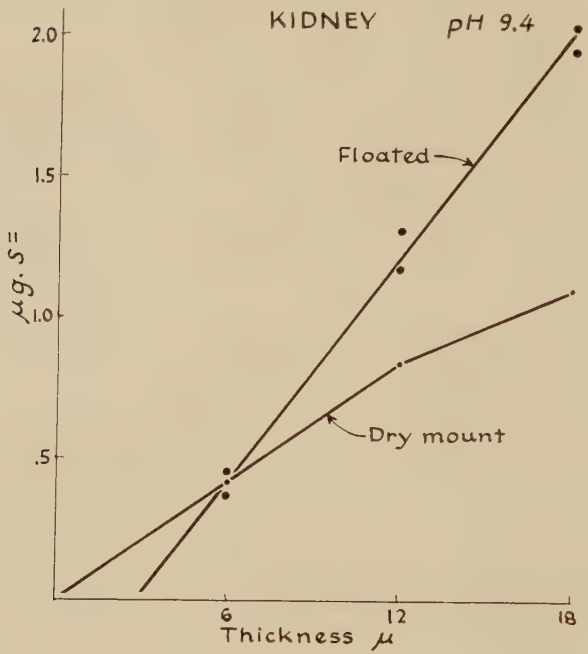

Fig. 7. Effect of floating acetone fixed sections on water when mounting them on slides. All sections hardened in alcohol after mounting on slides.

the origin. The floated sections give higher values which form a line intercepting the base line considerably to the right of the origin.

The intercepts and the slopes of the curves obtained indicate that the result of floating the acetone fixed sections prior to alcohol hardening is some further loss of enzyme (either by destruction or extraction), but also an increased overall activity of what enzyme remains in thicker sections. Since a swelling of the tissue is involved during flotation, one may assume that hardening of the swollen tissue gives a higher activity by virtue of the precipitation of a wider-meshed coagulum with increased availability of enzyme for contact with substrate. It also affects the conversion of phosphate to sulfide. The thinner sections stretch more extensively than do the thicker ones and perhaps there is proportionately more destruction than in thicker sections. This may contribute to the increased slope for the floated sections, figure 7.

On such a basis one can interpret the "fixing" action of acetone and alcohol on alkaline phosphatase as precipitating a coagulum in which some of the enzyme is destroyed, some of it left active, and some left in a poten-

TABLE III

Sulfide content of kidney sections pretreated as indicated.

Control Value	100± 8
100 per cent Methyl Alcohol	46± 7
50 per cent Methyl Alcohol	75± 6
10 per cent Formol (1 hour) washed in water 1 hour	83± 4
10 per cent Formol (1 hour) washed in water 24 hours	85± 2
1 per cent Acetic acid	118±10
1 per cent Nitric acid	0± 2

tially active form but occluded from the substrate by the coagulum. The results of treatment with water fit this explanation for the acetone coagulum. Similar effects on the alcohol coagulum were obtained when sections which had been acetone-fixed and alcohol-hardened were swelled by treatment with 1 per cent acetic acid for 15 minutes. (See Table III.)

The flotation effect may have other explanations. It is conceivable that inhibitors of phosphatase are removable by water from acetone-fixed preparations, but not from alcohol-fixed preparations. It can be noted in the sections concerned that the red cells lose more color in the sections treated with water before alcohol hardening than do those in hardened sections. Presumably other substances have corresponding solubilities. It may also be argued that some of these substances may play a role in the binding of the reaction products involved in the procedure.

2. Effects of reagents applied to sections before incubation.

Sections were processed according to the standard procedure through the step of hardening in 80 per cent alcohol. They were then treated either for 15 minutes with absolute methyl alcohol, 50 per cent methyl alcohol, 1 per cent acetic acid, or 1 per cent nitric acid, or for one hour with 10 per cent formol.

After rinsing in water the sections were incubated for one hour at 30° C and processed in the usual way for sulfide determinations. The results are shown below in table III.

Methyl alcohol used as an initial fixative destroys most of the phosphatase activity. The effects of methyl alcohol on phosphatase like the effects of ethyl alcohol, are considerably less destructive if the reagent is applied after a preliminary dehydration than if applied to fresh tissue. Thus the procedure of investigators (6, 16) who employed acetone fixation followed by alcohol is confirmed as giving higher values than preliminary treatment with alcohol despite the objections of Lison (12).

DISCUSSION

The choice of a histochemical technique for the localization of enzyme activity presents a dilemma. The selection of optimal conditions for quantitative studies on enzymes is closely bound up with the criteria for maximal activity. Maximal activity of a preparation depends in great part on the attainment of maximal accessibility of enzyme to substrate and this is most readily attained with both in solution. Alkaline phosphatase exists largely as a desmoenzyme which is extracted with difficulty from fresh preparations and is essentially unextractable from alcohol hardened sections. Maximal precision in localizing the enzyme requires minimal solubility of the enzyme. Thus the ideal fixation precludes maximal enzyme activity unless the sections were so thin as to bring all active enzyme to the surface of the structures in which they are bound. Failing this, the maximal contact between enzyme and substrate is attained with that fixative which gives the most permeable coagulum of structures containing the enzyme. It would appear that alcohol used as an initial fixative produces a denser, less penetrable coagulum than does acetone. Alcohol applied to tissues previously dehydrated in acetone produces a hardened coagulum of greater penetrability to substrate than that produced by initial alcohol fixation. There is presumably an additional direct action of acetone and alcohol in denaturing the active groups of the enzyme. This denaturant action is also a function of the state of hydration of the enzyme when the reagent is applied. We do not have sufficient data to assess the relative significance of the direct denaturing versus permeability aspects.

Determination of blank values. When the sulfide which is measured, is used as an index of the enzyme content of a section it is necessary to account for all non-enzymatic contributions to the sulfide value. Substances absorbing calcium, lead, or hydrogen sulfide or preformed phosphates can contribute to the non-enzymatic sulfide.

1. Incubation in a substrate containing an inhibitor may fail to completely block the enzyme (as in the case of fluoride in some acid phosphatase preparations), or may abolish the reaction by affecting the solubility of the reactants.
2. Incubation in a reaction mixture lacking the hydrolysable substrate component may lead to significant differences in ionic strength and composition.
3. Inactivation of the section by heating (if effective) or by chemical treatment may be expected to change other components of the section to some extent. Nitric acid will give complete inactivation but also remove calcium salts.
4. Some preformed substances such as calcium phosphate can be removed prior to determination of enzyme activity by judicious use of buffers of the effect of the

treatment on the enzyme is known. Accordingly there may exist some uncertainty as to the validity of the "blank" values. An alternative procedure for determination of non-enzymatic sulfide or phosphate is possible in some instances providing 1. a few nearly identical sections are available, 2. the assay procedure is strictly quantitative, 3. if two sections of different thickness give values forming a straight line passing through the origin. This checks points 1 and 2 but will include non-enzymatic as well as enzymatic contributions.

Now if three nearly identical sections of the same thickness are incubated for 3 time periods (30, 60 and 90 minutes) in a substrate in which the enzyme is stable; and if the sulfide values for these three points lie on a straight line; then the point of intercept at zero time indicates the amount of non-enzymatic sulfide to be accounted for in visual assessment. It does not indicate its location.

The enzyme must be fully hydrated before the incubation period starts and the longest time used must be short enough to preclude a changing rate of sulfide formation. The method will not give quantitative values for the amount of bone salt present but will measure the extent to which such salts contribute to sulfide deposition. The chief value of the method lies in excluding the non-enzymatic sulfide when the plot of the points passes through the origin.

SUMMARY AND CONCLUSIONS

1. A method is described for the measurement of the amount of sulfide deposited on sections which have been processed for the demonstration of phosphatase.
2. Using a standardized histological procedure on kidney cortex sections the amount of sulfide deposited was found to be directly proportional to section thickness at least through 12 μ.
3. In sections of given thickness the amount of sulfide deposited per unit time of incubation was constant for a period of two hours but decreased thereafter. Various reasons for the decreased rate are discussed.
4. The amount of sulfide formed is not a full measure of the amount of phosphate precipitated. Under one set of conditions only 40 per cent of the phosphate was accounted for as sulfide but the percentage was quite constant.
5. By treating acetone fixed sections with water, prior to hardening in alcohol, as in flotation experiments on slides, the amount of sulfide formed

after processing is increased. This result is explained in relation to the increased contact between enzyme and substrate after such treatment. Hardening of acetone fixed sections in alcohol causes a decreased enzyme activity as measured by direct quantitative assay but results in an increased deposition of sulfide in microscopic preparations. The disparity is discussed.

6. Some values are given for the effects of methyl alcohol, formol, acetic and nitric acids on phosphatase activity of sections previously fixed in acetone and hardened in alcohol. Brief treatment with acetic acid produces an activation of the phosphatase as measured by sulfide deposition.

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SOME NEW OBSERVATIONS ON THE ANIMALIZATION OF THE UNFERTILIZED SEA URCHIN EGG

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In the sea urchin blastula and gastrula the most animal part of the ectoderm is occupied by a high cylindrical epithelium with long stiff cilia forming the apical ciliated tuft. In the larvae this material constitutes a part of the ciliated band, this being above the mouth broader and thicker than elsewhere. The ciliated band consisting of cylindrical cells surrounds the oral field. The rest of the middle ectoderm is with some less important exceptions formed by flat cells as well as the vegetal ectoderm. Still more vegetally the three well defined parts of the endoderm follow: the hind-gut, the mid-gut, and the fore-gut. In the early blastula and still earlier stages the mesoderm material, the primary and secondary mesenchyme is situated vegetally to the fore-gut material. Thus in the blastula stage and earlier there is along the egg axis a series of presumptive organ rudiments, the proportions of which are within certain limits reproduced in the normal development. This is according to the well known "gradient hypothesis" of Runnström accomplished by the cooperation of two antagonistic principles located with their highest intensity at the animal and the vegetal pole respectively and gradually decreasing along the egg axis (cf. comprehensive review of Lindahl, (5)).

As early as 1892 Herbst found that treatment during certain periods of the development with lithium ions causes a displacement of the boundaries between the presumptive organ rudiments in the direction of the animal pole. As larvae treated in this way acquire a more vegetal appearance than normal ones, such larvae were characterized as vegetallized (3). However, it appeared possible to induce just the opposite effect, that is a displacement of the boundaries between the primitive organ rudiments in the direction of the vegetal pole, by treating the eggs before fertilization under aerobic conditions with ions presumably promoting a swelling (3, 4). In larvae

developed from eggs subjected to a treatment of this kind, the ciliated tuft usually dominates and the endoderm has become in greater or less extent transformed into ectoderm. Larvae of this type were called "animalized." Essential features of the procedure are 1) treatment with sulphocyanide or iodide ions and 2) conditions enabling intense oxidative processes. The treatment with sulphocyanide may be performed in natural sea water. As it is often necessary to extend the treatment over several hours, the tendency of the eggs to get over-ripe, which counteracts the animalizing effect, has to be checked. This is effected by the use of artificial sea water lacking Ca^{++} instead of normal.[1] As to the oxidative processes it was found (3) that low O_2-pressure as well as KCN and CO-O_2-mixtures in the dark check the processes in the cells causing animalization. Further evidence in this direction was brought by Runnström and Thörnblom (8), showing that addition of the oxidizing agent pyocyanine to the culture in a striking way promotes animalization.

Intending to study each of the two factors involved in animalization, the action of the SCN ions and the oxydative processes, we had first to take into consideration a suggestion advanced by Runnström and Thörnblom (8). They state that experiments in which the animalizing treatment was performed during the day very often gave positive results, whereas treatment started in the afternoon and carried out during the night were positive in rather few cases. "From these results it seems possible that light influences the animalization; the action of light may be an indirect one. The light may influence the bacterial growth." Referring to the results of Tyler, Ricci and Horowitz (12) on the relation between bacterial contamination and life-span of unfertilized sea urchin eggs, Runnström and Thörnblom write "A closer study will perhaps reveal that failure of the animalization is due to contamination with bacteria which promote over-ripeness."

To investigate the influence of light upon animalization we have carried out a number of experiments in which we also intended to study the influence of pyocyanine. As, however, our pyocyanine preparation turned out to be toxic in the concentrations needed, this part of the experiments will be left out here.

The experiments were performed with the eggs of females collected from two different localities within the Roscoff area in Brittany, Beclem and St. Efflam. The eggs from these two localities behaved quite differently during the summer 1948. At the first mentioned place the sea urchins may be collected by hand at very low tide and are at other times caught with a net, whereas at St. Efflam they are living

[1] That lack of Ca-ions counteracts over-ripening in sea urchin eggs was shown by Lindahl (4) and Schechter (9).

TABLE I

August 2, 1948. Eggs from a Beclem female treated for 10 hours. 500 larvae examined in each culture two days after fertilization.

		Animalized, per cent		
		With endoderm	Without endoderm	Total
Control	in light	0.6	0.2	0.8
	in the dark	0.4	0.6	1.0
Sulphocyanide	in light	8.4	20.8	29.2
	in the dark	6.6	46.0	52.6

in more shallow water. This may be the reason of the earlier breeding of the Beclem urchins than of the St. Efflam ones.

The experiments were performed in the usual way, the unfertilized eggs being treated for varying lengths of time 1) with Ca^{++} free sea water as a control and 2) with a mixture of 90 ml Ca^{++} free sea water +10 ml isotonic solution of NaSCN (0.54 M.). Then the eggs were washed with natural sea water and fertilized. All transferring of the eggs from one medium to another was effected with a small hand-driven centrifuge. Only eggs capable of being fertilized to 100 per cent were used. The cultures were kept in petri dishes of 10 cm diameter containing rather few eggs to avoid crowding. The experiments were performed during the day. All cultures were prepared in duplicate, one being kept in bright diffuse daylight[1] during the animalizing treatment and the other in the dark.

The results will be demonstrated by two typical experiments, one of which was performed with material from Beclem (Table I), the other with material from St. Efflam (Table II).

As seen from Table I some few animalized larvae were even found in the control cultures (5, p. 222). Among the animalized larvae the type with endoderm is more numerous, especially in the light culture, than has usually been the case, and those without an endoderm very frequently had a skeleton and an unpaired medial anal arm (Fig. 1). Thus the single larvae showed a less advanced animalization than has usually been observed.

Contrary to this the St. Efflam material is rather resistant to the animalizing treatment. Because of the small number of animalized larvae in the St. Efflam material, we have not specified to which of the two groups they belong. It was noticed, however, that the appearance of the specimens which were not animalized varied remarkably, some being normal plutei,

[1] Resting immediately next the pane of a big window facing to the north.

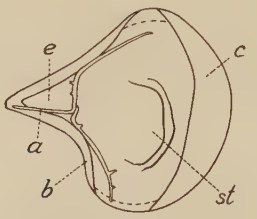

Fig. 1. Intermediary type of animalized larva of *Paracentrotus lividus*. a unpaired anal (post-oral) rod, b ciliated band, c cylindrical epithelium of ciliated tuft, e unpaired anal (post-oral) extension, st stomodaeum. 240/1.

others showing abnormalities in the skeleton, the arms and the apex. As there may be a relation between the action of light and the frequency of normal and abnormal plutei the frequencies of these two categories were established.

The type of larva frequently occurring in the animalized cultures with the Beclem material having a medial anal extension was totally lacking.

From these two examples the following may be concluded.

(1) Diffuse daylight inhibits animalization instead of favouring it. The same also was the result in all experiments with pyocyanine.
(2) The eggs taken from St. Efflam females are much more resistant to the animalizing treatment than those obtained from Beclem females.
(3) In the cultures with easily animalized eggs intermediate forms are frequent whereas they are rather scarce in cultures resistant to the animalizing treatment (4, p. 224).

Rothschild (7) has recently stated that light inhibits respiration in the unfertilized egg of *Psammechinus miliaris*. It must, however, be emphasized that the composition and intensity of light in his experiments and in ours are rather different as he uses a carbon arc as source of light, the distance of which from the illuminated eggs is not stated. Nevertheless the statement of Rothschild is of great interest as oxidative processes play an important role in animalization. On the other hand light has exercised a favourable influence on the development of the eggs which were not animalized in the St. Efflam experiment (Table II). The many small abnormalities appearing in the plutei of this experiment in all probability depend upon "over-ripeness." Thus the process provoking the state of "over-ripeness" seems to be checked by light.

It is very difficult to give any probable explanation of the strange result reported by Runnström and Thörnblom on the difference between their

TABLE II

August 6, 1948. Eggs from a St. Efflam female treated for 10 hours. 400 larvae examined in each culture.

		Per cent		
		Animalized	Normal plut.	Abnorm. plut.
Control	in light	—	67.5	32.5
	in the dark	—	0.5	99.5
Sulphocyanide	in light	—	92.7	7.3
	in the dark	8.8	87.2	4.0

night and day experiments other than that they have used St. Efflam material for their night experiments and Beclem material for their day experiments. The senior author has very often performed animalizing experiments during night with positive results.

The difference in resistance to the animalizing treatment between the eggs from the two localities is very striking. In our opinion the action of the sulphocyanide ions allows certain oxidative reactions to proceed which normally are not possible. If these processes would affect certain essential groups of an enzyme the activity of which is after fertilization indispensable for the realization of the vegetal morphogenetic principle, animalization should result. This would mean that the SCN-ions cause a swelling of the relevant protein involving such structural changes that the groups under consideration, being protected as long as the proteins are in a more dehydrated state, may be oxidized. However, we desist from speculation on the possible mechanisms underlying these changes. Herriott (1) reviews the literature on oxidation of enzymes and other native proteins.

As to the groupings being possibly oxidized during the treatment -SH deserves most attention but tyrosine and tryptophan are also oxidized under certain conditions. It has recently been shown (10, 11, 2) that saccharase and insulin are inactivated by tyrosinase and polyphenol-oxidase respectively, the inactivation depending probably on an oxidation of tyrosine residues.

If the picture sketched above has any relevance one would expect the enzyme protein indispensable for the vegetal principle and other proteins in the egg cell to be more easily swelled (or dissolved) under the influence of sulphocyanide or iodide ions in eggs from Beclem than in eggs from St. Efflam. To investigate this hypothesis unfertilized eggs were collected from

Table III

Total nitrogen in freeze-dried egg samples determined according to *Parnas* (1938).

	Egg sample mg.	Kjeldahl N mg.	per cent
Beclem	25.1	2.14	8.5
St. Efflam	38.5	2.75	7.1

females from the two localities with the aim of comparing the solubility of the egg proteins in isotonic NaI.[1] The Beclem material was prepared on July 31, the St. Efflam material on August 4, 1948.

The sea urchins were cut equatorially and the ovaries transferred with a horn spoon into filtered sea water, where the eggs were shed. The suspension was passed through bolting silk and washed several times in a slow hand-driven centrifuge to free the eggs from oocytes and leucocytes. The eggs were tested for fertilizability and gave 100 per cent fertilization. They were freed from their jelly coats with acid sea water and freeze-dried at $-70°$ C. and lower temperatures. The two dried samples were made homogeneous by being pulverized in a mortar and samples taken for nitrogen analyses (Table III).

The powder was suspended in ice cooled 0.54 M. NaI-solution and stirred mechanically for 4 hours at 0° C., the pH of the solution being regulated to 7.0 and buffered by addition of 3.0 ml. 0.2 M. phosphate buffer to 100 ml. NaI-solution. Experiments with the two materials were carried out simultaneously. After the extraction the samples were centrifuged at 10,000 r.p.m. in an angle centrifuge (radius 8 cm.) for 15 minutes. Of the clear solutions 5.0 ml. were taken for Kjeldahl analysis giving the amount of dissolved N-compounds. 10.0 ml. were transferred into a centrifuge tube, precipitated with 3.0 ml. 15 per cent solution of trichloracetic acid, the tubes left ice-cooled for 18 hours and centrifuged as described above for 15 minutes.[2] The clear solutions were decanted and the precipitates transferred into micro combustion tubes for nitrogen analysis, the obtained values representing the acid precipitable nitrogen compounds. Finally 5.0 ml. of the supernatant liquid were analysed for nitrogen giving the amounts of free amino acids and low molecular peptides.

[1] The I^- was used instead of the more effective SCN^- to avoid interferences with the nitrogen analyses.

[2] Generally the content of the tubes was then slightly coloured by iodine.

TABLE IV

Egg samples, the N-contents of these and the amount of liquid used for dissolving the egg proteins.

		Mg. egg substance	Mg. total N	Ml. 0.54 M. NaI-solution
Beclem	I	65.3	5.37	16.3
	II	100.8	8.59	25.2
	III	165.7	14.14	41.4
St. Efflam	I	80.2	5.73	20.1
	II	66.7	4.76	16.7
	III	98.6	7.05	24.7

TABLE V

Nitrogen-fractions as per cent of total nitrogen.

	Beclem			St. Efflam		
	I	II	III	I	II	III
Soluble N-compounds	71.5	90.1	81.5	59.8	61.5	65.6
N precipitable with trichloracetic acid	38.6	52.1	43.0	(22.8)	29.7	31.0
Non-precipitable N	30.7	38.5	37.6	28.7	31.8	32.9
Precipitable + non-precipitable N	69.3	90.6	80.6	51.5	61.5	63.9

Three different samples were submitted to this procedure, whereby the relation between the quantity of egg substance and the volume of the iodide-solution was kept constant (Table IV).

Comparing in each column the number at the bottom of Table V with that at the top provides a good control of the reliability of the analyses. Only in the first sample of the St. Efflam series is there discordance between the two values. Most probably a part of the precipitate has been lost when the supernatant was decanted.

From Table V it is obvious that the solubility of the egg proteins in 0.54 M. NaI is greater for the Beclem than for the St. Efflam material. The difference depends in the first place upon the fraction precipitable with trichloracetic acid but also to a lesser extent on the non-precipitable fraction. Experiments with the aim of detecting differences in the composition of free amino acids or peptides of low molecular weight are in progress, as well as analyses of reactive protein SH-groups at different stages of animalization.

The significance of the stated lower frequency of slightly animalized larvae in cultures with a high resistance against the animalizing treatment is not easily understood.

SUMMARY

The influence of bright daylight on the animalization of the unfertilized egg of *Paracentrotus lividus* by exposing it to SCN-ions in Ca^{++}-free sea water under aerobic conditions has been studied. The processes underlying animalization were decidedly inhibited by the light (Table I and II). It was found during the summer of 1948 that eggs taken from females from two different localities, Beclem and St. Efflam in the Roscoff area of Brittany, behaved very differently against the animalizing treatment, those from Beclem being easily animalized whereas those from St. Efflam were rather resistant. The proteins of the Beclem eggs turned out to be more soluble in icecooled 0.54 M. NaI than those of the St. Efflam eggs (Table V). In the cultures obtained from eggs not very resistant to the animalizing treatment, slightly animalized larvae were more frequent than in cultures with more resistant eggs.

The authors wish to express their hearty thanks to the authorities of the Biological Station of Roscoff, Prof. G. Teissier, P. Drach and Dr. Ch. Bocquet for their kind support of our work.

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STUDIES ON ISOLATED CELL COMPONENTS

I. NUCLEAR ISOLATION by DIFFERENTIAL CENTRIFUGATION

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The problem of the isolation of cell components in quantity has been approached in several ways. For the nucleus the methods have included acid-pepsin digestion of the cytoplasm (22), hemolysis of nucleated erythrocytes (1, 33, 19), and recently the differential centrifugation of mechanically disrupted cells. In preparing tissues for differential centrifugation the following procedures have been employed: *1.* dessication (5); *2.* ultrasonic disintegration (16); *3.* freezing and thawing (11); *4.* heating to 51° C (38); and 5. treatment with citric acid (30, 11, 21, 4). In most of the above methods the tissue is killed before nuclear isolation. Other workers have mechanically broken up living tissue in saline (17, 12) or sucrose solutions (28) and have then isolated the nuclei by differential centrifugation in these solutions. For further references on methods the recent review of Dounce (11) may be consulted.

The method of choice for the isolation of nuclei will be dictated by the purposes of the experiment, as emphasized by Dounce (11). For the investigation of the physiological and colloid chemical potentialities of the isolated nucleus, which is the aim of the present studies, it becomes necessary to employ procedures which will furnish quantities of pure nuclei in a state resembling the intracellular condition as closely as possible. This means giving attention to such factors as electrolyte composition, tonicity, the maintenance of pH near neutrality during and following extraction, etc. Since technics previously devised for other purposes were not suitable in these respects, modifications have been introduced. A simple method is presented which permits the isolation of rat liver nuclei in a state of purity satisfactory for various colloidal and biochemical studies and in

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a condition optically similar to nuclei within the living cell. The isolation procedure constitutes a starting point for an examination of the requisite conditions for the maintenance of nuclei *in vitro*.

METHODS

Before presenting the detailed method for nuclear isolation, mention may be made of certain general procedures which we have found useful in the preparation and centrifugal fractionation of homogenates of living tissues. An excellent general discussion has previously been given by Hoerr (17).

1. *General considerations*

Temperature.

The maintenance of low temperature during all operations is essential. At room temperatures mitochondria in liver brei fragment rapidly (2), and nuclei become granular and irregular (35). Also loss of enzymes from nuclei occurs on warming and is illustrated by the finding that nuclei prepared by the earlier method of Dounce contained no catalase, whereas nuclei prepared with a refrigerated centrifuge had a high catalase content (11). The use of a refrigerated room is only a partial answer to the problem since suspensions are warmed during centrifuging. It has been observed, for example, that the temperature of distilled water in 15 ml centrifuge tubes rose from 5.8° to 10.5° during 20 minutes centrifuging at 1,170 × g in an International Centrifuge Size 2 operated in a cold room at 5.2° C. For work involving centrifugation for long periods at high speeds a refrigerated centrifuge is almost indispensable.

Preparation of homogenates.

Rats of the Osborne-Mendel strain or hamsters (*Cricetus auratus*) were stunned by a blow on the head and rapidly decapitated. Possible effects of anesthetics are thus eliminated. The liver was flooded with cold 0.15 M NaCl and perfused with the same solution. The organ was then excised and homogenized in cold medium (usually 5 ml of medium per gram of fresn liver). The entire procedure was carried out in a refrigerated room at 5° C.

The disruption of cells has usually been accomplished by pressing the tissue through bolting silk after preliminary grinding (6); by the use of the Waring Blendor l.c. or similar device (10); or by the use of tight fitting grinders (14, 31, 25, 36). Kneading through bolting silk is not satisfactory when a high percentage of cells must be ruptured; and the Waring Blendor has been found too drastic for use with unfrozen tissues and may denature certain proteins (29). Temperature control has also been found somewhat difficult with the latter technique (10).

Simple glass, plastic, or rubber grinders operated by hand (25, 36) will give no rapid and complete homogenization of brain, liver, and kidney. For most work pestles with a diameter of approximately 0.8 inches fitted in test tubes ten inches in length have been used. A study of the grinding characteristics of eleven pestles

TABLE I

The pH of Rat Tissue Homogenates.

All homogenates consisted of 1 gram of tissue (fresh weight) per 5 ml. of medium. The entire procedure was carried out in a refrigerated room at 5° C.

Preparation	pH of medium	pH of homogenate immediately after preparation	pH of homogenate after one hour at 5° C
Perfused rat liver in:			
Distilled water	5.78	7.1	7.02
Sucrose-phosphate[1]	7.01	7.02	6.98
Isotonic KCl	5.98	6.92	6.82
Unperfused rat kidney in:			
Distilled water	5.78	6.92	6.84
Sucrose-phosphate[1]	7.01	7.05	6.92

indicates that the pestle diameter should be about 0.2 mm less than the tube diameter. About seventy slow up and down strokes of the pestle will disrupt nearly all the cells in 8 to 10 grams of rat liver as the tissue passes between the pestle and the tube wall. Since the clearance is many times the thickness of the liver cell, contact with both grinding surfaces is probably not necessary. With less clearance gases are likely to be withdrawn from the solution by the upward movement of the pestle. All homogenates are strained through bolting silk or cheesecloth before centrifugation to remove connective tissue.

Hydrogen ion concentration.

Since the pH of the cytoplasm of certain individual cells has been shown to fall from about pH 6.8 to 5.4 on injury (8, 37) homogenates prepared in unbuffered media may be expected to be decidedly acid. This is not the case with rat liver, even though a large portion of the blood buffers has been removed by perfusion. Table I shows that the pH of liver and kidney homogenates in unbuffered solutions is close to neutrality and falls no more than 0.1 pH unit in the course of an hour at low temperature. Greene et al (13) reported similar results but stated that rabbit kidney formed acid homogenates; and Hoerr (17) found that guinea pig liver homogenates in neutral water reached a pH of 5.6.

Centrifugal procedures.

The principles involved in differential centrifugation are simple and well known. However, they are sometimes disregarded, making the purification of material more laborious than necessary. A few practical applications of the principles which have proved helpful in isolating nuclei may be noted.

[1] Composition listed under section on solutions.

In centrifuging a tube filled with a homogenate the separation of components in initial centrifugings will inevitably be incomplete except for the lightest fraction, even though the sedimentation velocities of the various components are quite different. This follows from the fact that a portion of all the components is in the centrifugal end from the beginning and only the last to sediment can be obtained in pure form. Also, the particles near the centrifugal end of the tube are subjected in many centrifuges to twice the force exerted on the top of the tube—a factor of some importance in cases in which one wishes to sediment whole cells or nuclei and leave mitochondria in suspension. Repeated differential centrifugations will eventually give relatively pure preparations, however.

A much more efficient procedure consists in layering the homogenate over a denser medium in the centrifuge tube. In the sucrose method for isolating mitochondria (18) approximately 10 ml of homogenate are layered over one ml of a denser sucrose solution. Ideally, a very thin layer of homogenate would be placed at the top of a very long centrifuge tube. The components would then have ample opportunity to separate according to their individual sedimentation velocities. In practice, layers one or two centimeters in thickness layered on top of a denser medium in 50 ml conical centrifuge tubes have been used to separate nuclei from whole cells and mitochondria as described below. Care must be taken to avoid mixing of the layers during acceleration and deceleration of the centrifuge. This has been accomplished with the International refrigerated centrifuge, model PR-1, by attaching an accessory rheostat which permitted the transition between the vertical and horizontal positions of the centrifuge tubes to occur over a 30–60 second period.

Microscopic observations.

Identification of nuclei in homogenates of fresh liver tissue presents no problem with dark field or phase, but with the light microscope individual clear, hyaline nuclei may be difficult to see. For this reason we have found it convenient to use methylene blue dried on slides from an alcohol solution for routine examination of samples of the homogenate during the course of purification of the nuclei. Whole cells, mitochondria, and nuclei will all take up the dye. It should be pointed out that methylene blue and other similar basic dyes cause clumping of the mitochondria and precipitate cytoplasmic proteins of the fresh liver homogenate.

Solutions.

Our present knowledge of the constitution of cytoplasm is too fragmentary to permit us to duplicate its inorganic composition. As a beginning we used a modification of a solution containing KCl, NaCl, and sodium citrate which Chambers found maintained chromosomes of Chironomus larvae in good condition (8). This solution was unsatisfactory due to clumping of cytoplasmic constituents. It was felt advisable to eliminate the chloride ion since it is found only in very low concentration in the liver cell (32). Calcium was not included in view of the marked effects produced even in low concentrations on the cytoplasm (8) and on the isolated nucleus (34, and below). Since potassium and phosphate predominate in the cytoplasm (15) these ions are used here. Bicarbonate is included in a somewhat lower concentration than found in fresh liver (9).

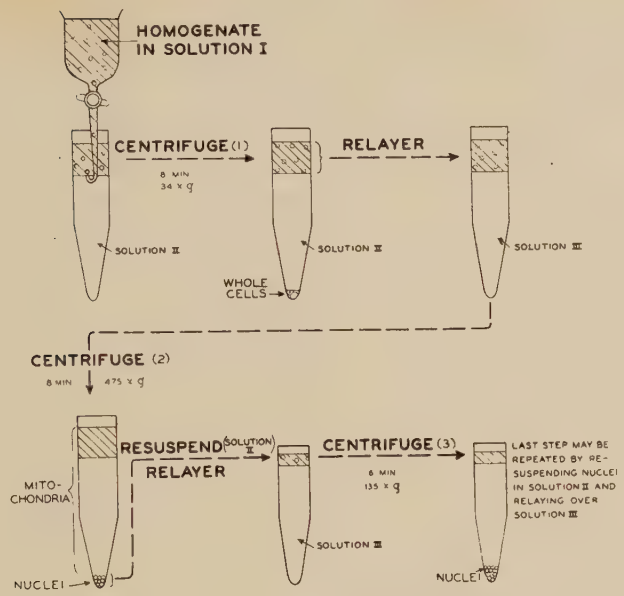

Fig. 1. Nuclear isolation procedure.

In devising solutions for the present purposes the following factors were taken into account: tonic composition, freezing point depression, density, and pH. Sucrose solutions, though satisfactory for the preservation of mitochondria (18) are not entirely suitable for the isolation and maintenance of nuclei.

The following solutions have been used in the present work:

1. *Solution I.* 0.0094 M KH_2PO_4; 0.0125 M K_2HPO_4; 0.0015 M $NaHCO_3$; 0.145 M sucrose. Sp. gr. 1.023 at 25° C; 0.7 × isosmotic; pH 7.1.
2. *Solution II.* Same salt concentration as solution I, sucrose 0.218 M. Sp. gr. 1.031 at 25° C; 0.9 × isosmotic.
3. *Solution III.* Same salt concentration as solution I, sucrose 0.272 M. Sp. gr. 1.040 at 25° C; 1.2 × isosmotic.

The salt concentration and pH are constant in all three solutions. The density requirement for differential centrifugation has been met by variations in sucrose concentration, which in turn alters the tonicity somewhat. The osmotic values are referred to 0.15 M NaCl. It must be emphasized that these solutions are to a certain extent arbitrarily devised and may need to be altered as better criteria of unclear preservation are worked out.

Sodium chloride (0.15 M) has been used for perfusion. Inasmuch as heparin releases nucleic acids from isolated cell components (3) and citrate removes calcium and magnesium from perfused tissues (20) these anticoagulants have not been employed.

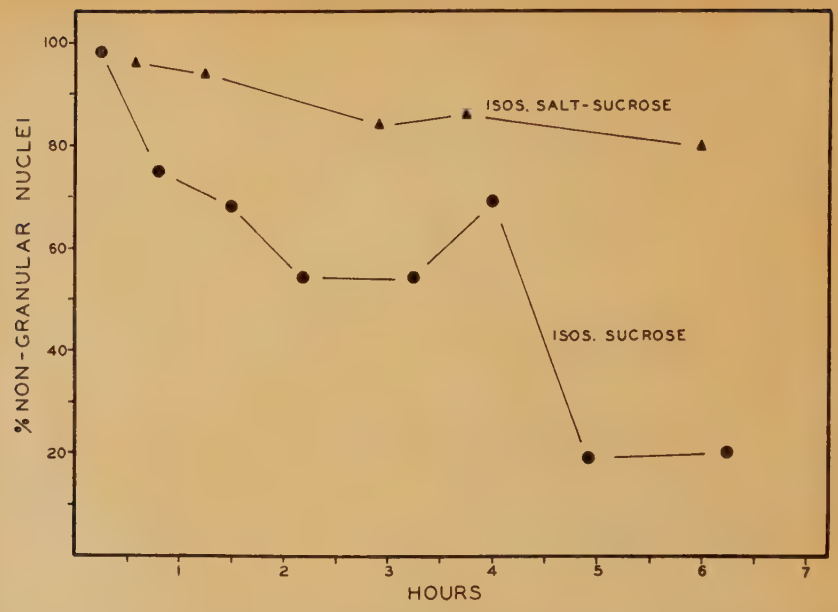

Fig. 3. Increase in granularity of isolated rat liver nuclei with time. Each point represents the results of examination of approximately 100 nuclei. Temp. 0°. Sucrose-salt mixture: 0.06 M sucrose; 0.03 M KH_2PO_4; 0.07 M K_2HPO_4; pH 7.1. Isosmotic sucrose, 0.29 M; pH 6.3. Solution I gave better preservation than either of these solutions.

2. *Nuclear isolation procedure*

The centrifugal procedure is illustrated in Fig. 1. Centrifugal forces are calculated at the bottom of the tube.

1. *Homogenization of rat liver.* 5 ml of solution I added per gram fresh liver. Homogenized with 70 strokes in hand homogenizer. Strained through 10XX bolting silk or four layers of cheesecloth.
2. *Removal of whole cells.* 15–20 ml of homogenate carefully layered over 30 ml of solution II in a 50 ml conical centrifuge tube (see Fig. 1). Centrifuged 8 minutes at 34 × g, observing the precautions for acceleration and deceleration mentioned under centrifugal procedures above.
3. *Sedimentation of nuclei.* Entire upper layer (15–20 ml) removed by sucking up into layering device shown in Fig. 1 and relayered over 30 ml of solution III. Centrifuged 8 minutes at 475 × g. Entire supernate discarded.
4. *Resedimentation of nuclei.* Nuclei resuspended in solution II (5 ml) and layered over solution III in a 15 ml centrifuge tube with a bulb pipette. Centrifuged 6 minutes at 135 × g. Step 4 repeated twice when highest purity is required, centrifuging 6 minutes at 135 × g and 6 minutes at 53 × g.

The entire procedure can be carried out in about 90 min.

Fig. 2. Fresh rat liver nuclei showing nucleoli and absence of granulation.

RESULTS

Microscopic observations.

The isolated nuclei are similar in optical appearance to those observed in the fresh intact cell, though very slightly larger on the average. They are smooth hyaline spheres free of granulation when observed under the light microscope (Fig. 2). One to three nucleoli are readily visible. Under dark

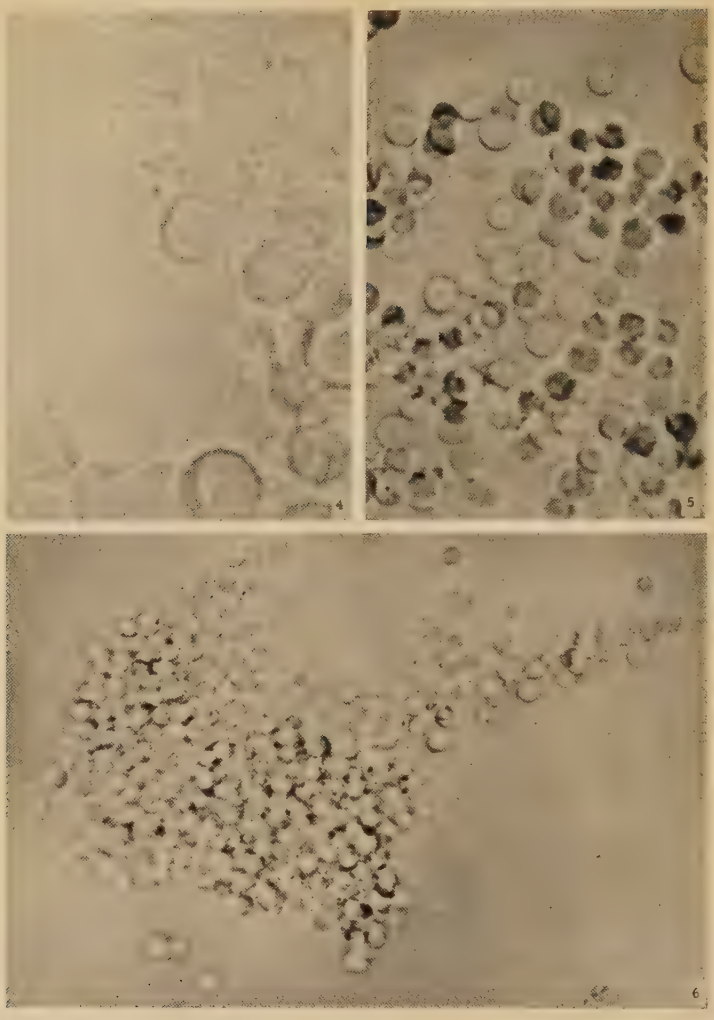

Fig. 4. Fresh rat liver nuclei isolated by procedure described in text. Photographed by direct illumination in Solution II described on page 54. Note occasional mitochondria appearing as small dark bodies between nuclei.
Fig. 5. Preparation similar to that shown in Fig. 6, but photographed by oblique illumination.
Fig. 6. Isolated rat liver nuclei showing clumping which occurs in neutral solutions.

field the nuclei are either optically empty or show a few small granules. With methylene blue a uniform light stain is observed, while the nucleoli stain somewhat more darkly.

The preparations are of a high degree of purity. A few mitochondria are trapped between the nuclei in the final preparation (Figs. 4–6) and an occasional whole cell is found. The ratio of whole cells to nuclei in an average preparation is considerably less than 1 : 100. Exact counts of nuclei are difficult to make since the nuclei form large aggregates (fig. 6).

As a measure of the maintenance of normal appearance at low temperature, determinations were made of the percentage of nuclei showing distinct granulation in a salt-sucrose solution and in pure sucrose at intervals after the completion of the isolation procedure. The results are shown in Fig. 3 (see legend). In isosmotic sucrose-salt mixture approximately 80 per cent still maintain their normal appearance after six hours at 0° C. The results are similar for solutions of widely different tonicity. At 37° granules quickly develop. The percentages of nuclei of normal appearance are smaller in pure sucrose solutions at 0°.

Ion effects on the isolated nucleus will be considered in a separate communication. Reference will be made here only to the surface deformations brought about in isosmotic NaCl and KCl solutions (2) and to a marked and rapid shrinkage resulting from the addition of 0.001 M $CaCl_2$ to nuclei in an isosmotic salt-sucrose solution (see legend for Fig. 3).

Nucleic acid analyses.

The nuclear yield in the isolation procedure has been determined by analysis of the desoxyribonucleic acid (27, 7) in the original homogenate and in the purified nuclei. The yield is about 5 per cent. This can be increased by a briefer centrifugation in Step 2 but at the expense of an increase in the proportion of whole cells.

Analysis of 5 preparations of nuclei gave a protein-desoxyribonucleic acid ratio of 5.1 with a range of 2.7 to 8.9. DNA was determined by the diphenylamine reaction and protein was calculated from total nitrogen after removal of lipids and nucleic acids. Protein-DNA ratios of 6.5 to 20 have been obtained in absorption studies of nuclei stained by the Feulgen technique (24). The lower values found in the present study might be thought to indicate protein loss during isolation—and such may indeed occur. However, in view of the uncertainty of the determinations by optical absorption (26, 23), protein loss from nuclei isolated as described above cannot be inferred. The problem may perhaps be attacked by the differential centrifugal isolation and chemical analysis of fixed nuclei.

DISCUSSION

The primary purpose of the present investigation has been to obtain in quantity nuclei which retain certain of their normal properties. Using the method here described, hyaline, nongranular nuclei resembling those within the living cell have been isolated. However, optical similarity offers no guarantee that changes may not have occurred. In fact, there is some reason to believe from the slight size difference of intra- and extracellular nuclei that changes have taken place during isolation. Indeed, it would be surprising if the simple salt-sucrose solution used in isolation could satisfactorily substitute for the cytoplasm and leave the nucleus unchanged. Nonetheless, the method of isolation here described makes available for biochemical and physiological study nuclei which have been subjected to relatively gentle treatment and are essentially free from cytoplasmic constituents. The preparation has proved useful in studies of nucleoprotein gels using the solutions mentioned (3). For other studies new media will be needed, their composition depending upon the purposes of the experiment. This should require no basic change in the method of isolation.

SUMMARY

A method is presented for isolating nuclei of rat and hamster liver in a high state of purity and in a condition optically similar to nuclei within living cells.

The isolation procedure consists in the homogenization and differential centrifugation of fresh liver at 0–5° in a salt-sucrose solution buffered at pH 7.1. By layering the material to be centrifuged over a relatively large volume of a slightly denser solution the purification can be carried out in 4 or 5 centrifugations. The entire procedure can be completed in about 90 minutes. The yield as determined by measurements of desoxyribonucleic acid is about 5 per cent.

The solutions contain KH_2PO_4, K_2HPO_4, $NaHCO_3$, and sucrose. The sucrose concentration is varied to give density differences required for layering. Salt-sucrose solutions maintain a large proportion of the isolated nuclei in a nongranular condition for 6 hours at 0°. Pure sucrose is less satisfactory for maintenance.

The protein-desoxyribonucleic acid ratio for the isolated nuclei averages 5.1 with a range of 2.7 to 8.9.

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NUCLEUS-CYTOPLASM RELATIONSHIPS IN THE ACTION OF ULTRAVIOLET RADIATION ON AMOEBA PROTEUS[1]

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Since we have no means of observing nuclear behavior in the absence of cytoplasm, the role of the nucleus in the economy of the cell must be evaluated by observations on cells where the nucleus has been removed or experimentally altered. One tool for experimental alteration is radiation. While it is hardly the case that radiation effects are restricted to the nucleus, the liminal effects often express themselves through nuclear phenomena. It is very difficult to detect physiological or biochemical effects with small radiation doses that do affect the cytogenetic system and cell division. The so-called "killing" of microorganisms by irradiation is an effect on reproductive processes such that the "killed" cells survive for long times and may seem normal by ordinary physiological criteria. If radiation damage sufficient to stop reproduction actually had no effect on the non-reproductive physiology of the cell, it might be concluded that the nuclear apparatus was, like the "germ plasm" of the multicellular organism, unconcerned with the maintenance of the individual. We know that this is not the case from the observation that enucleated cells or cells whose further reproduction has been inhibited by irradiation do ultimately die. The data on what happens to such cells is very meagre, and the purpose of the present investigation was to explore the relation between normal nuclear behavior, as evidenced in reproduction, and the maintenance of the cell. Certain of the present data are of interest in connection with the problem of the participation of nucleus and cytoplasm in radiation effects on the cell.

The material chosen was *Amoeba proteus*. The advantages of this form are the large size and the relative ease with which the cells may be cut into

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[2] Atomic Energy Commission Postdoctoral Fellow, 1949–50. Part of this investigation was performed during tenure of this Fellowship.

nucleate and enucleate halves, or the nucleus removed micrurgically. The disadvantages are the low division rate and the unavailability of a synthetic culture medium.

Ultraviolet radiation of λ 2537 Å was employed. The specific problems dealt with are:

1. Effects of irradiation of the medium.
2. Effects of doses below the sterilization level on subsequent divisions.
3. The magnitude of the sterilization dose.
4. Survival of sterilized cells as compared with enucleate cells.
5. Effect of removal of part of the cytoplasm on radiation sensitivity.
6. Effect of radiation on enucleate fragments.

METHODS

Clones of *Amoeba proteus* were established from a stock obtained from the General Biological Supply House, Chicago. In all of the experiments reported, the cells used were descended from a single individual.

In the absence of a pure culture method, a culture system adapted from Brandwein (4) was employed, and it was found that reproducible data on division rate could be obtained by attention to detail. The method was found to be effective for large cultures and for small cultures in depression slides (straight sides, 16 mm diameter, 3 mm deep). The balanced salt solution contained 120 mg NaCl, 3 mg KCl, 4 mg $CaCl_2$, 2 mg $NaHCO_3$ and 5 ml Sorenson phosphate buffer of pH 7.0 per liter. Glass-distilled water was essential. The solution was layered over a film of 0.75 per cent agar. In large cultures rice grains were added. The food organism was *Chilomonas paramecium*. For the depression slide cultures a drop of a fairly dense population of *Chilomonas* was added. In some cases *Paramecium* was also present. The cultures were maintained at 20–21° C.

A Westinghouse WL 782L-30 Sterilamp was employed. Most of the ultraviolet output of this lamp is at 2,537. Incident ultraviolet energy was measured by means of the Hanovia Ultraviolet Meter. The manufacturer's calibration was employed. All irradiations were at a rate of 36 ergs/mm²/second. Dose was controlled by varying the time of exposure.

For each experiment a group of about 50 cells was washed free from food organisms and debris and placed in the inorganic medium in a Stender dish. The depth of medium was 1 cm. After the dishes were positioned under the lamp, sufficient time was allowed for the amoebae to settle, attach, spread, and commence active movement, then they were irradiated. Controls were similarly handled but not placed under the lamp. Immediately after irradiation single amoebae were transferred to depression slides prepared as described above. Generally, 5 cells from each lot of 50 was followed.

Where cell fragments were studied, the general procedure was the same. Previous to irradiation, the amoebae were cut into equal halves, free hand, by means of glass needles, and nucleate and enucleate fragments separated.

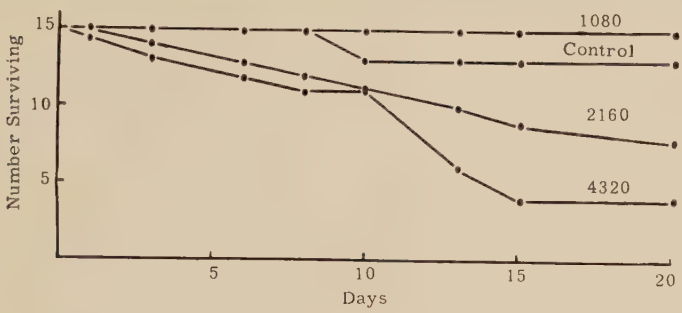
RESULTS

Effect of irradiation of medium

In these experiments, the depression slides prepared with agar and inorganic medium were irradiated before introduction of food organisms and amoebae. Doses up to 65,000 ergs/mm^2 were applied and *Chilomonas* and amoebae then added immediately. There seemed to be no clear-cut immediate effect on the amoebae. On the other hand, the behavior of the *Chilomonas* definitely indicated the presence of irradiation products in the medium. The movement of these flagellates was slowed down and many of them settled to the bottom of the culture. Generally they resumed activity after about 15 minutes. In most experiments the division of the amoebae was normal. Recent experiments have called attention to the fact that (16) ultraviolet irradiated culture media may have effects on micro-organisms that are qualitatively similar to effects of direct irradiation. The effects are attributed to the formation of peroxides. Nothing in the present investigation tends to prove or disprove the hypothesis that radiation effects in general may be mediated by decomposition products of water in the cell (17). Our experiments merely indicate that the effects described below are not obtainable when the medium alone is first irradiated, even when the medium includes agar.

Effect of irradiation on multiplication and survival of intact amoebae

In these experimental series, 15 individuals were isolated following each dose and the cultures followed for 20 days. Figure 1 plots the survival of the organisms in one typical series. "Survival" is defined merely as the physical presence of the organism and survivors reported at a given time may include dead cells that have not yet disintegrated. Doses below 1,000 ergs/mm^2 were tested but the results are not represented because these cells behaved no differently from controls. Doses above 8,000 ergs/mm^2 caused rapid cytolysis. But, in the dose range represented the effect observed is a limitation of the life-span of the irradiated cells. The effect is dose-dependent. While all of the cells survived a dose of 1,080 ergs/mm^2 and very few survived a dose of 4,320 ergs/mm^2, about half of the cells survived a dose of 2,160 ergs/mm^2. Not only the number of cells surviving, but the span of survival of those which died is obviously dose dependent. It will be noted that the observations extend over only 20 days.



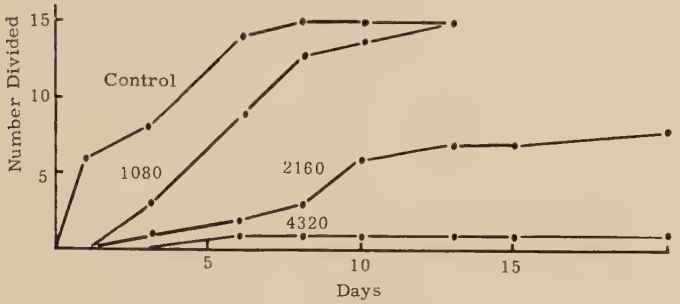


Fig. 1. Survival of whole amoebae after irradiation. Numbers over curves indicate incident ultraviolet dose in ergs/mm².

Fig. 2. Division of whole amoebae (same groups as in Fig. 1) after irradiation. Ordinates represent number of originally isolated cells which have accomplished one division.

Fig. 2 gives the data on division in the same population. The ordinates represent the number of cells in each group which had divided. It is now apparent why the 20 day period of observation was sufficient. By the twentieth day practically all the cells had either divided or died. In fact, of the 60 cells isolated for culture, only 3 had survived without division by the twentieth day. Observations on such cells showed that they invariably died, none surviving beyond the 29th day. There is, therefore, a very clear relationship between what we shall call "sterilization" and survival. An irradiated cell will either divide within about 20 days, or will die. On the other hand, those cells which do divide once will produce progeny capable of further divisions. As will be shown in the next section, recovery is generally complete. In the experiments shown in Figure 1 and 2, a few of the irradiated cultures did die after division, but since this took place also in unirradiated controls, it is attributed to experimental incidents rather than to an effect of radiation that expressed itself after division.

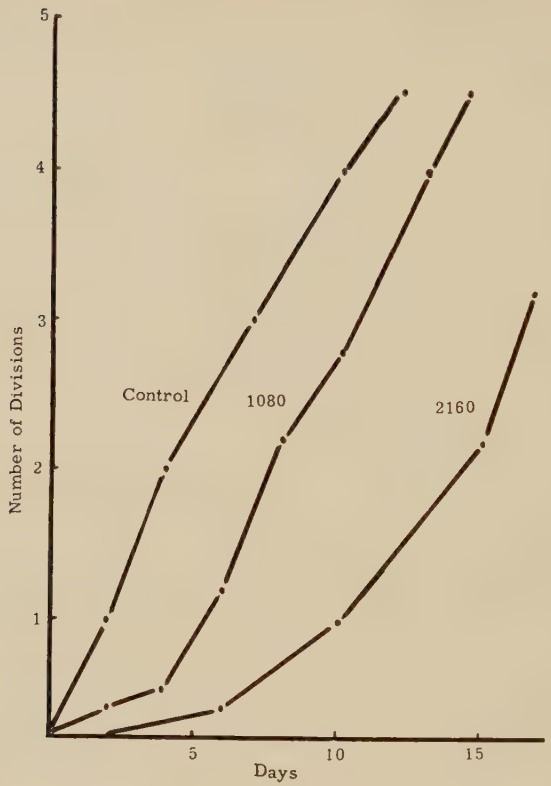


Fig. 3. Division rate following irradiation. Each curve is based on counts of the progeny of 5 isolated amoebae. Ordinates represent total number of divisions accomplished. The number of divisions accomplished during each time interval was computed on the basis of the number of individuals present at the beginning of that interval. In this procedure, the curves are not cumulatively affected by the death of individual cells.

Division rate following irradiation

Fig. 3 gives data on the division rate of amoebae irradiated, then isolated. The ordinates represent the average number of divisions per cell. Each curve is based on the progeny of 5 cells. The number of cells present in the culture at a given time is divided by the number present at the previous observation to compute the number of divisions accomplished. This computation eliminates those cells which died in the course of the experiment.

It is seen that the effect of doses below the sterilization level is to delay the first divisions following irradiation. The average delay is clearly dose-

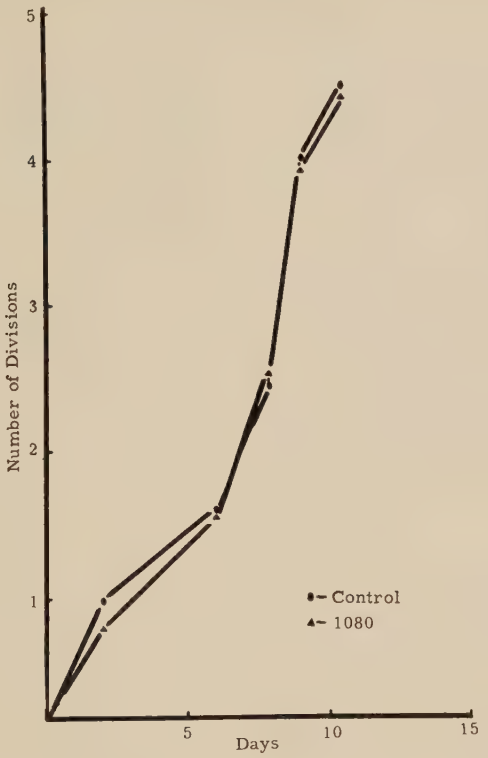


Fig. 4. Long term recovery from effect of radiation dose below sterilization level. Control curve represents division rate of individuals isolated from parent clone. Experimental curve represents division rate of individuals isolated 19 days after irradiation from clone established by isolation of an irradiated individual from the same parent clone. Radiation dose: 1080 ergs/mm^2. Ordinates present total number of divisions accomplished.

dependent. Following the period of delay, the progeny tend to resume the division rate of the controls. In general, recovery is complete after one or two divisions. Figure 4 shows the division rate of a group of cells isolated from the progeny of an amoeba that had received 1,080 ergs/mm^2 19 days previously. The division rate is identical with that of a control group isolated from the parent clone. The progeny of irradiated cells have been cultured for many months in a search for permanent effects of the irradiation. We have been able to detect no effects other than the initial delayed divisions in such experiments.

DISCUSSION—PART I

The first effect of the irradiation—delayed division—is one that has been described for many other cases. It is of interest to compare the magnitude of the dose required in the case of Amoeba with that observed for other types. Giese (11) has summarized some of the data in the literature. The effective doses for various types of cells range from less than 100 ergs/mm^2 to several thousand.

The effective dose for delaying division in Amoeba is in the same range —1,000–2,000 ergs/mm^2—as that for other Protozoa that have been studied and for similar effects on invertebrate eggs. If there is any uniform trend in the whole body of data, it seems to be toward decreasing sensitivity with increasing cell size. This is conceivably of interest in relation to the problem of the role of the cytoplasm in these effects. As will be shown later, the sensitivity of Amoeba may be increased merely by amputating part of the cytoplasm.

The delay in division caused by the radiation is completely reversible. What is of greatest interest in the present results is the effect when the dose has been extended to the point of irreversibility: the sterilization which is often referred to as "killing."

The coincidence between "sterilization" and the loss of capacity for long term maintenance of the cell is striking. We have found in lengthy experiments in which food was limited, that *A. proteus* may survive for many months without division. But a radiation dose which deprives the cell of the *capacity* to divide also limits its survival to about 20 days or less even under favorable nutritional conditions. The effect seems to be entirely sharp; those cells which can achieve a division following irradiation, however long the delay, produce a normal progeny while those which do not divide within about 20 days die. Thus there seems to be a decisive relationship between the mechanisms responsible for further division and those responsible for long-term maintenance. It does not seem likely that the identity of the dose-dependence of the two effects is accidental.

The behavior of the sterilized cells is in superficial respects comparable to that of enucleate cells. A conspicuous difference is that the sterilized cells may survive as long as 20 days while enucleate cells seldom survive longer than 10 days. In the cases both of enucleation and sterilization by radiation we are dealing with the factors determining the long-term survival of the cell. There is no evidence of removal or inactivation of systems essential for current metabolism. The long-term effect suggests that the nucleus

(and the component of the reproductive mechanism which is affected by radiation, probably associated with the nucleus) is not concerned with the activity of the biochemical mechanisms carrying out the instantaneous work of the cell, but rather with syntheses essential for their replacement. We assume that they undergo "turnover," although information on the turnover of metabolic machinery, as contrasted with that on metabolic intermediates, is practically non-existent.

Amoeba as experimental material offers certain important possibilities for further analysis of these questions. The question, whether transplantation of an unirradiated nucleus would restore the capacity of a sterilized amoeba to divide might be answered by employing the technique of Chaton and de Fonbrunne (6). Current unpublished work in this laboratory show the feasibility of preparing sufficient quantities of enucleate amoebae for quantitative studies with the Cartesian Diver and with isotope techniques. An analytical comparison of the effects of enucleation and radiation is thus possible.

Finally, it should be mentioned that we have detected no permanent changes resulting from irradiation nor any consistent production of giant cells or multinucleate cells such as are common in irradiated populations of microorganisms (14, 12). We have obtained binucleate amoebae which did not divide further, but the binucleate condition is not too uncommon in populations of Amoeba.

Effect of radiation on multiplication and survival of nucleate half amoebae

Fig. 5 and 6 present data of a typical series of experiments carried out in essentially the same manner as those given in Fig. 1 and 2, except that in this case nucleate half amoebae were irradiated. The fragments were prepared by cutting with glass needles, free-hand, as described. It is evident that the results are qualitatively the same as those in the experiments on whole amoebae. Above a given dose, some of the cells were sterilized and did not divide within the 23-day period of observation. The proportion of cells sterilized increased with dose. At the end of the period, practically all of the cells had either divided or died; of the 75 cells observed, only three had survived without dividing, and these ultimately died. In the case of those cells which did divide, the effect of irradiation was again a delay in the first division and the duration of the delay was dose-dependent. It would not seem that the results were influenced by the necessity for the half-amoebae to restore their lost cytoplasm before division, for it

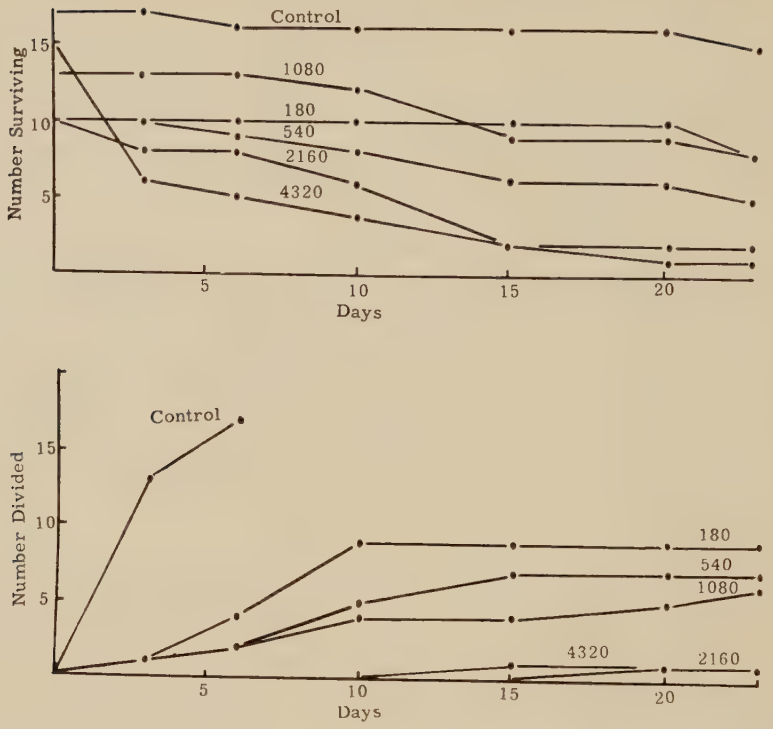


Fig. 5. Survival of nucleate half-amoebae after irradiation. Numbers over curves represent incident ultraviolet dose in ergs/mm². In this experiment, varying numbers of individuals, as indicated by the zerotime ordinate, were isolated after each dose.

Fig. 6. Division of half-amoebae (same groups as in Fig. 1) after irradiation. Ordinates represent number of cells in each group that have accomplished one division.

is noteworthy that the controls in this series divided just as rapidly as the controls in the experiments on whole amoebae.

However, amputation of half of the cytoplasm distinctly affected the results quantitatively. The dose required to produce a given effect was very much decreased. The half-amoebae were more than twice as sensitive, as whole amoebae. Whereas the dose for 50 per cent sterilization of whole amoebae was in the neighborhood of 2,000 ergs/mm², the same degree of sterilization for half amoebae was achieved with only 540 ergs/mm². A dose of 180 ergs/mm², whose effect on whole aoebae could not be detected, caused an appreciable delay in the first division of half amoebae. It is obvious that amputation of part of the cytoplasm has a real effect on radiation actions that are generally considered to be mediated by the nucleus.

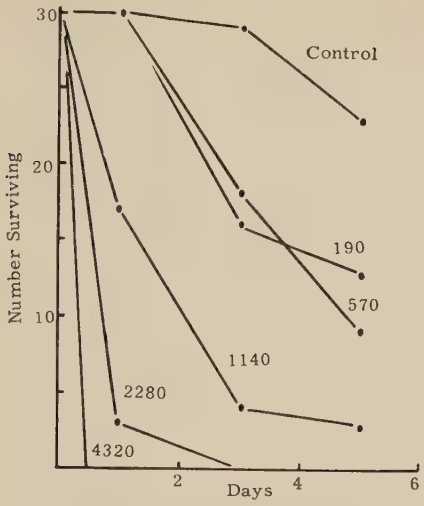


Fig. 7. Survival of enucleate half amoebae following irradiation. In this experiment, 30 control fragments and 30 fragments for each dose were followed.

Effect of irradiation on survival of enucleate half-amoebae

Since the enucleate half-amoebae do not divide, their radiation sensitivity can be measured, within the limitations of the present experiments, only in terms of survival. Fig. 7 shows the results of a series in which 30 fragments were observed for each dose. The data show a marked radiation sensitivity by this criterion. A dose of only 190 ergs/mm^2 resulted in the disintegration of more than half of the fragments in 5 days, during which period 80 per cent of the unirradiated controls survived. In general, higher doses shortened the span of survival even more. Because of the limited survival of the controls, it is not certain that a valid comparison can be made between these data and the data on the fragments containing nuclei. The main significance of these observations is that they demonstrate definite photochemical effects on the cytoplasm alone at low doses, effects which must be taken into account in the evaluation of the results previously presented.

DISCUSSION—PART 2

The data on ultraviolet effects leave little doubt that both enucleate and nucleate half-amoebae are more sensitive than whole amoebae. The observations on the survival of enucleate fragments show that these are sen-

sitive in terms of immediate cytolytic effects even at low doses. It can hardly be questioned that we are observing direct photochemical action on cytoplasmic constituents. The data on the division delay and sterilization of nucleate halves would indicate that even in the case of nucleus-associated phenomena, cytoplasmic reactions enter.

The whole question of the nucleus versus cytoplasm as the site of action of radiation has been the subject of considerable discussion (8, 11). For ultraviolet effects, the case for the nucleus has depended on the action spectrum and the fact that in the case of gametes characteristic cleavage abnormalities may be produced by the irradiation of spermatozoa alone (10, 15). Giese (8) and Blum and Price (2) have presented cogent arguments for the participation of both nucleus and cytoplasm in ultraviolet effects that express themselves as cleavage inhibition.

The cytolytic effects of relatively low doses on enucleate fragments does indicate a direct photochemical damage. The presence of a nucleus protects the cell fragment against this action, as shown by the survival data even on those nucleated fragments which were sterilized. This in itself would be merely another example of the dependence of morphogenetic processes—in this case repair of photochemical damage—on the nucleus (1). The one additional fact is that even in the cell in which further division has been prevented by irradiation there is still some evidence of a constructive role of the nucleus.

The effect of amputation of half of the cytoplasm, increasing the radiation sensitivity both with respect to cell division and with respect to survival, is more difficult to account for if, as the whole body of literature suggests, the nucleus plays a decisive role as the point of attack of radiation. The most obvious explanation would be that the cytoplasm serves to shield the nucleus from radiation. This simple explanation is difficult to maintain in the case of amoeba. Nucleate amoebae, whether whole cells or fragments, tend to attach to the surface and spread out in a thin layer. The layer of cytoplasm shielding the nucleus from radiation from above is not necessarily greater when the total volume of cytoplasm is greater.

Evidence on this point is presented in Fig. 8. These photographs were taken at 2537, with the quartz microscope. The technique was essentially like that of Lavin (13). Living whole amoebae and nucleate halves were photographed. The cells were allowed to settle on the quartz slide in a large drop of culture medium and were not covered. The posture of the amoebae was therefore that which obtained in the irradiation experiments. The poor quality of the photographs is a consequence of the thickness of

Fig. 8. Ultraviolet photomicrographs (λ 2537) of a whole amoeba and a nucleated half amoeba. Cells were photographed in uncovered drop of culture medium and were in active movement during exposure.

the cells and the fact that they were in active streaming motion during the exposure, but it is difficult to see any conspicuous difference in the average transmission of the whole amoeba and the nucleate half amoeba. The

photographic technique was such that differences in transmission should have been conspicuous. The emulsion employed was the Eastman Superspeed Ortho Portrait, which gives a linear relation between exposure and density over a broad range, and uniform exposure and development were attempted.

If the function of the cytoplasm is not merely to shield the nucleus, the radiation effect on the nucleus must depend in some way on processes in the cytoplasm. Moreover, the cytoplasmic contribution must depend on the absolute amount of cytoplasm. This result is not remarkable in terms of what we actually know of nucleus-cytoplasm relationships. The study of cell regeneration makes it clear that morphogenesis (which would include syntheses of specific cell structures) is nucleus-dependent. It is equally clear from the essentially negative results of the search for energy-yielding systems in the nucleus that the cytoplasm is probably responsible for the gross turnover of materials and energy (5, 7, 3). If we are dealing with a reversible action of radiation on the nucleus—and the merging of delayed division into sterilization indicates this to be the case in the present experiments—it is to be expected that some of the mechanisms on which the reversal depends will be in the cytoplasm. For a layer of the same thickness, the relative inactivation by radiation will be the same for all volumes, but a nucleus surrounded by the normal amount of cytoplasm will have at its service, after a given dose, a larger absolute number of surviving active units than a nucleus in half the normal cytoplasmic mass. Therefore, the half-amoeba might be expected to be more sensitive and to be sterilized at a lower dose by virtue of the deficiency in cytoplasmic mechanisms concerned with reversal of nuclear damage.

The sensitivity of the cell would thus depend on a three way interaction between (a) the radiation action on the nucleus, (b) the reversal involving cytoplasmic activities, and (c) the inactivation of the cytoplasmic systems on which the reversal depends. If the net consequence of this interaction was an irreversible damage to the nucleus, expressed as sterilization, then the surviving cytoplasmic mechanisms may maintain the cell for a period of time. But, if the nucleus is essential for replacement of cytoplasmic mechanisms, the survival is limited by the life-span of essential systems which cannot be replaced.

Whatever the precise explanation of the results of these amputation experiments, they do demonstrate a nucleus-cytoplasm interaction in radiation effects. Such a treatment is applicable only to cases of potentially reversible effects and may not apply to chromosomal alterations, identified

TABLE I

Summary of ultraviolet effects on intact amoebae and fragments.

Dose ergs/mm²	Effects		
	Whole amoebae	Nucleate fragments in per cent	Enucleate fragments in per cent
180–190	No effect	Delayed division	50 per cent disintegration in 3 days
540	No effect	Ca 40 per cent sterilization Delayed division of survivors	50 per cent disintegration in 3 days
1080	Delayed division	Ca 60 per cent sterilization	50 per cent disintegration in 1 ½ days
		Delayed division of survivors	85 per cent disintegration in 3 days
2160	50 per cent sterilization	Almost 100 per cent sterilization	50 per cent disintegration in 1 day
	Delayed division of survivors		100 per cent disintegration in 3 days
4320	Almost 100 per cent sterilization	Almost 100 per cent sterilization	50 per cent disintegration in 12 hours
	50 per cent disintegration in 10–15 days	50 per cent disintegration in 3 days	100 per cent disintegration in 24 hours
10,800	Immediate disintegration	Immediate disintegration	Immediate disintegration

by cytogenetic means. It may be that in the latter case, where the units affected by radiation are unique, it is possible to detect the residue of units whose radiochemical alteration is not reversed.

It would follow from all these considerations, as well as from the work of others, that the question of exclusive nuclear or cytoplasmic radiation effects may be without meaning.

SUMMARY

1. The effects of ultraviolet irradiation on the division rate, capacity to divide, and survival of whole amoebae and nucleate and enucleate half cells have been observed. The overall effects are summarized in Table I.

2. The first effect of irradiation of whole amoebae and nucleated half amoebae is a delay in the divisions immediately following. This is com-

pletely reversible; later progeny of the irradiated amoebae have a normal division rate.

3. Amputation of half of the cytoplasm greatly increases the radiation sensitivity as measured by delayed division or by the dose required for permanent inhibition of division (sterilization dose).

4. Amoebae that have received the sterilization dose may survive for 20–30 days but not longer.

5. The survival time of enucleate fragments is very much reduced by small (200–500 ergs/mm^2) doses of ultraviolet radiation.

6. It is concluded that the overall radiation effect may have both nuclear and cytoplasmic components.

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NUCLEIC ACIDS IN RELATION TO CELL DIVISION IN *LILIUM LONGIFLORUM*[1]

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The nucleic acids owe a great part of their interest to their association with chromosomes, and other organelles of the cell, and to their supposed role in cell division and growth. A quantitative knowledge of their relationships to the mitotic cycle, and to the processes of cell growth and cell differentiation, would therefore be expected to throw some light on a number of fundamental problems.

In principle, quantitative data on nucleic acid contents of cells at various stages of development may be obtained by either of two methods, microscopic spectrophotometry of individual cells or portions of cells, or chemical analysis of large numbers of cells in the same stage of development. Several workers have made studies by microscopic absorption methods, either in the ultraviolet, in the region of purine absorption of the nucleic acids (3, 14, 15), or in the visible after reaction of the nucleic acids with basic dyes or the Feulgen reagent (14, 15, 16, 19).

On the whole, however, the data obtained so far by microscopic absorption methods do not appear to be entirely reliable. Danielli (5) has offered a general critique of these methods. Pollister and Ris (14) have emphasized the need for suitable blank determinations. Commoner (4) has called attention to the influence which molecular orientation of the absorbing substances may have on extinction. The Feulgen reaction has been examined critically by the Stedmans (18), Ris and Mirsky (16), and others. Kaufmann (10) has demonstrated some of the precautions which are necessary

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in the methods which utilize enzymatic hydrolysis in conjunction with basic dyes.

Biochemical assay methods are more accurate. However, the methods which are available at the present time require amounts of material several orders of magnitude greater than are present in a single cell. To obtain analytical data which will have meaning in terms of the mitotic cycle or processes of cellular differentiation, one should have means of obtaining large numbers of cells all in the same stage of development, and means for estimating the number of cells in each analyzed sample, so that the analyses could be expressed in biological terms, *i.e.* per cell, rather than on a gravimetric basis, *i.e.* per unit weight of tissue.[3] It would be convenient if the cells were discrete, rather than in a consolidated tissue, or could be separated, so that their number could be estimated by hemocytometer methods. This has been done for isolated nuclei of animal tissues at one stage of development, namely, maturity (1, 12). To extend this procedure to various developmental stages of cells, a method of determining the developmental phase represented by a sample of cells would be required. The microscopic appearance of a cell is a sensitive index of its mitotic stage in the period from prophase to telophase, but it is well known that the conventionally recognized mitotic stages occupy greatly different lengths of time. The interphase, during which there are no good morphological criteria of developmental status, usually occupies a longer period of time than all the other stages combined. Consequently, if one wished to refer analytical data on dividing cells to a time scale on the basis of microscopic study of the cells, he would have little to go on during the extended interphase. In effect, one would not have a time axis available for a considerable part of the developmental period. It would therefore be convenient to have some other criterion of the developmental status of the cells than microscopic appearance.

The suitability of *Lilium longiflorum* for the study of biochemical and physiological aspects of meiosis and mitosis has been indicated by Erickson (7). Since the growth in length of the flower bud is exponential in nature from an early stage until shortly before anthesis, and since the exponential growth of various floral organs appears to be quite precisely correlated with the growth of the bud as a whole, the logarithm of bud length serves as an excellent index of the developmental status of a bud, or of the

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[3] We are grateful to Dr. Alfred E. Mirsky for his enthusiastic encouragement at the time when plans for this study were being formulated, and for his emphatic recommendation that analyses such as these be referred to numbers of cells.

organs contained within it. More specifically, a scale of logarithms of bud length may be used as a time scale in plotting data of various types obtained from a series of developing buds.

The development of pollen within the anther involves in the earliest stages, a succession of mitotic divisions of undifferentiated anther cells, which produce the microsporocytes. The diploid microsporocytes then divide by meiosis to produce haploid microspores, and these in turn undergo mitosis to form the two-celled pollen grains. Meiosis and the subsequent mitosis occur synchronously within all six anthers of a bud, and can be located at definite points on the logarithmic bud length scale. Since the microsporocytes, microspores and pollen grains are free cells[4], rather than a consolidated tissue, they can be readily removed from the anthers and freed from the surrounding wall and tapetal tissue. The cells can be suspended in a salt solution and samples withdrawn for counting. By scaling down the perchloric acid-extraction method for analysis of the nucleic acids described by Ogur and Rosen (13), it has been possible to carry out analysis for desoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) on samples of cells obtained from single flower buds.

The analytical results which are reported here cover a large part of the developmental period of the anther contents, including, 1. late meiosis, 2. the microspore interphase which occurs between meiosis and microspore mitosis, 3. the microspore mitosis, and 4. the pollen grain interphase between microspore mitosis and anthesis.

EXPERIMENTAL

Preparation of Material

Lilium longiflorum, var. "Croft" has been used, since the var. "Floridii," used previously, was not available at the time of this investigation. Plants were grown in the greenhouse during the spring of 1949. During the summer, when greenhouse temperatures were higher, plants were grown out-of-doors. The var. "Croft" differs from "Floridii" in producing larger flowers, among other things. Precisely how this size difference is reflected in the nature of the growth curves of floral parts, and their allometric relationships is not known, since the detailed growth analysis made of "Floridii" (7) has not been repeated. No essential differences are apparent. Cytologically, the cell divisions are shifted slightly on the bud length axis. Diakinesis and metaphase I of meiosis in "Croft" had their peak at 22.5 mm bud length, in both greenhouse and garden-grown buds, just as in "Floridii." Microspore mitosis

[4] Throughout this paper, the term "cell" is used for convenience, to refer to the individual microsporocyte, microspore or pollen grain, even though the latter actually consists of two cells.

occurred at 63 mm, and anthesis at about 180 mm, in the greenhouse-grown material and the later material from the garden, as compared with 57 mm and 150 mm, respectively, for "Floridii." The first buds from the garden had their mitotic peak at a somewhat smaller bud length and opened at lengths as low as 150 mm. This temporary disturbance (2 or 3 weeks) of the growth pattern perhaps accounts for part of the variability of the results reported below.

Buds, whose lengths were recorded, were dissected, and the anthers removed, weighed, and taken to a cold room (4° C) for further dissection. Each anther was cut in half transversely, and the cells transferred to a cold salt solution (0.21 M KCl, 0.07 M NaCl, and 0.007 M $CaSO_4$). This salt solution was used throughout the course of development after preliminary microscopic observation indicated bursting of pollen in sucrose solutions below 0.5 M. It was at least isotonic or hypertonic to the cells at all stages of development and approximated a balanced salt solution in terms of membrane stability. At bud lengths below 60 mm the cells are suspended free in the tapetal fluid and were readily squeezed out of the half-anther for transfer. At greater bud lengths the pollen is associated with a yellow oily material which leads to considerable clumping of the cells. The cells were transferred by scraping the inside of each anther locule, agitating the dissecting needle in the salt solution, and wiping the needle intermittently to remove the accumulated yellow material. It has been found in more recent work that this yellow material can be removed more easily by shaking the cell suspensions with a small amount of an organic solvent such as petroleum ether.

Each sample of cells was transferred to a 6.5 ml hematocrit tube containing the same cold salt solution, and washed three times by gravitational sedimentation, except in the earliest stages, where sedimentation was speeded by low speed centrifugation (about 200 rpm in a clinical centrifuge). Before each sedimentation the suspension was agitated by bubbling air through it at minimum pressure by means of a fine capillary tube which reached the bottom of the hematocrit tube. In the first sedimentation of each sample, the volume of sediment was recorded at one-min. intervals so that a sedimentation-time curve could be drawn. At the greater bud lengths the sediment consisted of two distinct layers. The lower layer, pale yellow in color, accounted for most of the material and accumulated in 4 or 5 min. The upper, darker yellow layer, which settled more slowly, was seen to consist of abnormally small, empty cells when examined microscopically. On the basis of the sedimentation-time curve, a time was chosen for subsequent washes so that the empty cells were discarded when the supernatant liquid was drawn off by aspiration through a capillary tube.

After the third washing the suspension was continuously agitated with the air stream while samples were withdrawn for counting. Since the cells sediment so rapidly, it was not possible to use the hemocytometer principle, in which the volume of the sample counted is derived from the dimensions of the counting chamber. Instead, samples were withdrawn quickly by means of a calibrated micropipette (usually 0.055 ml) and each was spread out on the surface of a 1×3 in. microscope slide, the entire surface of which was diamond-ruled into approximately 1 mm squares. The slides were then oven-dried and all cells were counted at low magnification either dry or after flooding the slide with aqueous methyl violet, and covering with a cover glass. From the mean of replicate counts the total number of cells

remaining in the hematocrit tube was calculated. The sedimented volume of the cells was also recorded. The supernatant liquid was again aspirated off and the cells were transferred with a minimum of cold 95 per cent ethanol to a small glass homogenizer, made by grinding a ball-tipped glass pestle into the bottom of a 1 ml test-tube. About 2 hrs usually elapsed between the dissection of buds and homogenization of the cells.

Chemical Methods

Each sample of cells was homogenized in cold 95 per cent ethanol and the homogenate was extracted according to the procedure described by Ogur and Rosen (13). Preliminary extractions of non-nucleic acid material were performed with cold acidulated ethanol, with boiling ethanol-ether, and with cold 2 per cent perchloric acid. The constituents of pentose nucleic acid (PNA) were extracted by treatment of the residue with 10 per cent perchloric acid at 4° C for 18 hours. Further treatment of the residue with 10 per cent perchloric acid at 80° C for 20 minutes extracted the constituents of desoxypentose nucleic acid (DNA).

These nucleic acid extracts were examined by microphotocolorimetric assay methods using the Beckman spectrophotometer. Quartz microcuvettes requiring 0.1 ml of material were employed and assay methods were scaled to levels of 0.1 μg of nucleic acid phosphorus per 0.1 ml. Ultra-violet extinction of the perchloric acid extracts was measured directly at several wave-lengths besides that of maximal absorption. Total phosphorus was determined by a modification of the Fiske–Subba Row (8) procedure; pentose and desoxypentose by modifications of the orcinol reaction (11) and diphenylamine reaction (6) respectively.

In the PNA extracts at several bud lengths, the difference in the ultra-violet extinction at 260 mμ and 300 mμ was in good agreement with the phosphorus found in these extracts, using samples of yeast nucleic acid as standards. Pentose assays yielded much higher values, as has been reported with root tip material (13) due to common constituents of plant tissues which interfere with the orcinol reaction.

In the DNA extracts at several bud lengths the ultraviolet extinction at 270 mμ gave good agreement with the phosphorus found. In the period of development between 30–40 mm bud length the diphenylamine reaction yielded satisfactory checks with the ultra-violet and phosphorus data based upon thymus and sperm samples of DNA treated under comparable conditions. In our early experiments during the latter period of development (above 40 mm bud length) substances interfering with the diphenylamine reaction were extracted with hot perchloric acid as evidenced by high values and displacement of the peak absorption from 600 mμ. In our more recent experience cell suspensions were washed with petroleum ether. After this treatment, it has been possible to obtain checks between the diphenylamine and ultra-violet assays in mature pollen.

In the majority of the runs described in this report ultra-violet extinction alone was used in the assay of nucleic acid extracts and the data calculated to PNA phosphorus (PNAP) and DNA phosphorus (DNAP) on the basis of analyses of isolated nucleic acid samples under conditions comparable to those used in extraction. An optical density of 0.350 was obtained at 260 mμ with a 1 cm light path, for 0.1 μg of PNAP in 0.1 ml of 10 per cent perchloric acid. In experimental runs, samples

of cells were chosen and dilutions, where necessary, were performed so that the optical densities read were of this general order of magnitude. Only 5 of the points reported are based on densities less than 0.200 and none on densities less than 0.100. Amounts of PNAP and DNAP calculated from ultra-violet data have been divided in each case by the number of cells in the sample, to express nucleic acid levels on a per-cell basis.

RESULTS AND CONCLUSIONS

Cell counts, hematocrit readings and total amounts of DNAP and PNAP for each sample are given in the first part of Table I. In the last three columns, the DNAP and PNAP contents per cell, and cell volumes are given. Some of these data are also presented in the figures.

In Fig. 1, the DNAP content of single cells, in $\mu\mu g$[5] has been plotted along the ordinate. Values of DNAP have been converted to values of DNA by dividing by 0.099.[6] On this basis, a second ordinate, giving values of DNA has been added to Fig. 1, and the graph may be read in terms of either DNAP or DNA. The abscissa is a logarithmic scale of flower bud lengths. Since logarithm of bud length is linearly related to time (7), this is equivalent to a time axis. If an exponential bud growth rate of 0.0765 is taken (7), the time interval between 25 mm. and 60 mm. bud length, for instance, is 11 days. The points at which meiosis (*i.e.*, diakinesis and metaphase I), microspore mitosis and anthesis occur are indicated by the shaded vertical lines.

Three analyses have been made of microsporocytes during late meiosis (below 25 mm. bud length). Since it was not possible to obtain accurate cell counts in this region, because of clumping of the cells, the values of DNAP per cell below 25 mm. are only approximate. Nevertheless, these values are about 4.8 times as high as those directly above 25 mm., where the microsporocyte walls have broken down and the microspores are free. The DNAP content of a microsporocyte at the end of meiosis must certainly be 4 times that of a newly-formed haploid microspore.

In the interval between meiosis and mitosis, the DNAP content of the microspores increases. This increase appears to be linear with respect to logarithm of bud length. Accordingly an equation of the form

$$Y' = a + b \ lnL_b$$

[5] This unit, a micromicrogram, appears to be of suitable magnitude for expressing the amounts of certain chemical substances contained in single cells. It is equal to mg $\times 10^{-9}$ (13), to $\gamma \times 10^{-6}$ (1) and to gm $\times 10^{-12}$.

[6] This factor has been used to permit comparisons with the data of other workers, pending more experimental work on the composition and molecular weight of the macromolecular nucleic acids of different tissues. A similar factor has been used to convert PNAP to PNA.

TABLE I

Nucleic Acid Analyses and Cell Counts of Microsporocytes, Microspores, and Pollen of *Lilium longiflorum*.

Run No.	No. of buds	Bud length (mm)	Total DNAP (μg)	Total PNAP (μg)	Hemato-crit (μl)	Est. cell No.	DNAP per cell (μμg)	PNAP per cell (μμg)	Est. cell vol. (μμl)
				Microporocytes.					
16	6	21.7	0.18	0.27		4,200	42.2	63.1	
15	3	22.2	0.34	1.61	10	21,000	16.0	75.3	470
26	1	22.6	0.94	0.94					
43	9	22.8	16.29	18.75	110				
27	1	24.6	0.64	1.25		37,000	17.0	33.4	
				Microspores.					
41	1	27.3	0.76	1.74	20	111,000	6.8	15.7	180
33	1	28.3	0.76	1.84	15	161,000	4.8	11.4	90
42	1	30.0	0.59	1.69	13	154,000	3.9	11.0	80
17	1	30.8	1.11	1.74	10	161,000	6.9	10.8	60
20	1	33.5	2.13	1.24	40	138,000	15.4	9.0	290
23	1	36.0	1.71	3.96	40	196,000	8.7	20.2	200
28	1	36.8	1.84	3.79	60	283,000	6.5	13.4	210
11	2	38.6	2.78	4.11	100	317,000	8.8	13.0	320
12	2	39.0	3.17	5.94	115	332,000	9.6	17.9	350
30	1	41.6	1.99	2.82	50	237,000	8.4	11.9	210
34	1	47.7	2.17	3.90	69	241,000	9.0	16.2	290
29	1	51.5	1.80	3.44	62	160,000	11.3	21.6	390
35	1	56.4	1.26	2.57	62	169,000	7.5	15.2	370
				Pollen Grains.					
13	2	63.4	3.05	9.66	145				
14	2	63.4	3.34	10.34	160	151,000	22.2	68.5	1060
18	1	63.8	1.72	2.88	60	61,000	28.4	47.5	990
21	1	68.0	2.64	5.34	100	93,000	28.2	57.2	1070
31	1	74.2	1.58	4.88	70	66,000	23.8	73.6	1060
32	1	79.9	2.30	7.81	115	101,000	22.8	77.6	1140
24	1	83.1	3.01	10.36	120	93,000	32.5	112.0	1300
9	1	91.7	3.14	9.31	180	118,000	26.5	78.7	1520
10	1	93.7	2.50	6.25	91	78,000	32.1	80.3	1170
36	1	98.8		7.26	88	63,000		115.6	1400
25	1	111.7	2.25	9.11	72				
37	1	122.6	1.16	4.57	45	34,000	34.2	134.2	1320
40	1	128.1	1.23	4.26	40	27,000	45.1	155.8	1460
22	1	133.9	3.04	6.61	65	53,000	57.5	125.2	1230
19	1	151.0	2.69	10.31		81,000	33.2	127.2	
39	1	179.6	3.44	16.11	130	95,000	36.3	169.6	1370
38	1	184.4	3.11	12.55	116	81,000	38.3	154.4	1430

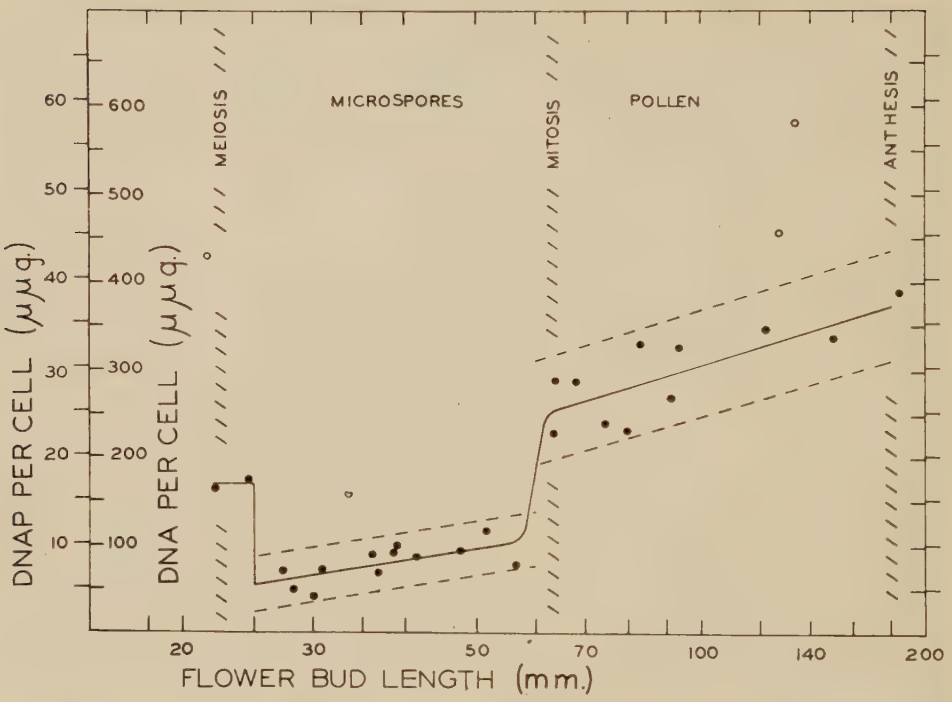

Fig. 1. DNAP and DNA content of developing microspores and pollen grains. Solid lines have been fitted to the data by least squares, with the omission of points shown as open circles. Broken lines indicate 95 per cent confidence intervals about the 2 fitted lines.

may be fitted to the data by the least squares method. (Y' is the calculated DNAP content of a microspore in μμg; a is a constant; lnL_b is the natural logarithm of bud length; and b is the rate of increase of DNAP content with respect to lnL_b.) Constants of equations of this type which have been fitted to the data are listed in Table II. For the period between meiosis and mitosis, the rate of increase of DNAP, b, is 5.98.[7]

[7] One point, shown as an open circle in Fig. 1, has been omitted in this calculation. If this point is included, a regression line is obtained, whose slope, b, is 4.51. The point in question deviates from this line by an amount which is 2.7 times the standard deviation about the line. In fitting the data on DNAP between 60 mm and 180 mm, two points, at 128.1 and 133.9 mm bud length, have been omitted in calculating the value of b shown in Table II. If they are included, they are found to deviate from the regression line by 1.1 and 3.7 times the standard deviation about the line, respectively. These three points are undoubtedly high because of unrecognized errors in the counting or analytical procedure. It seems reasonable to omit them. With these omissions, all of the regressions reported in Table II, for both DNAP and PNAP, are highly significant.

Another statistical aspect of these data may be mentioned. The cell counts, which were made in duplicate or quadruplicate for each sample, vary more than would be expected on the basis

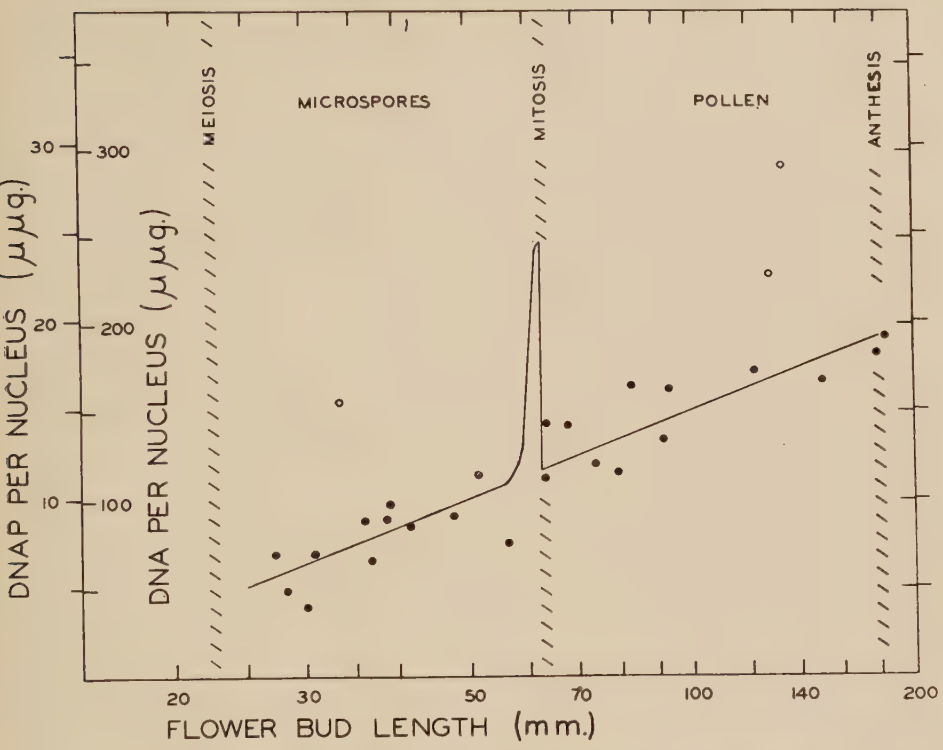

Fig. 2. DNAP and DNA content of haploid nuclei of microspores and pollen grains, fitted with a single straight line.

At the end of this period of increase the DNAP content of the microspores increases rapidly to more than twice the preceding value. This rapid increase appears to occur shortly before microspore mitosis, since mitotic figures have been seen only at 62–63 mm bud length. In the development of the pollen grains from the end of mitosis to opening of the flower, DNAP increases again, apparently in a linear manner. The rate of increase, *b*, omitting two high points[7], is found to be 11.44.

of random sampling. The combined χ^2 value for deviations of the counts from mean counts for each sample, is 265.8 with 45 degrees of freedom, and has a very low probability. If separate values of DNAP per cell are calculated from the analytical results, using separate counts, rather than the means of counts for each sample, the proportion of the total variance due to differences between replicate counts may be estimated. Using the data as represented in Fig. 2, the variance due to counts is significantly lower than the remaining variance about the regression line ($F = 3.74$, with a probability of about 0.001). It would be difficult to evaluate the variance due to analyses, but it seems unlikely that this is large. The largest part of the variance is probably connected with variables in the culture of the plants.

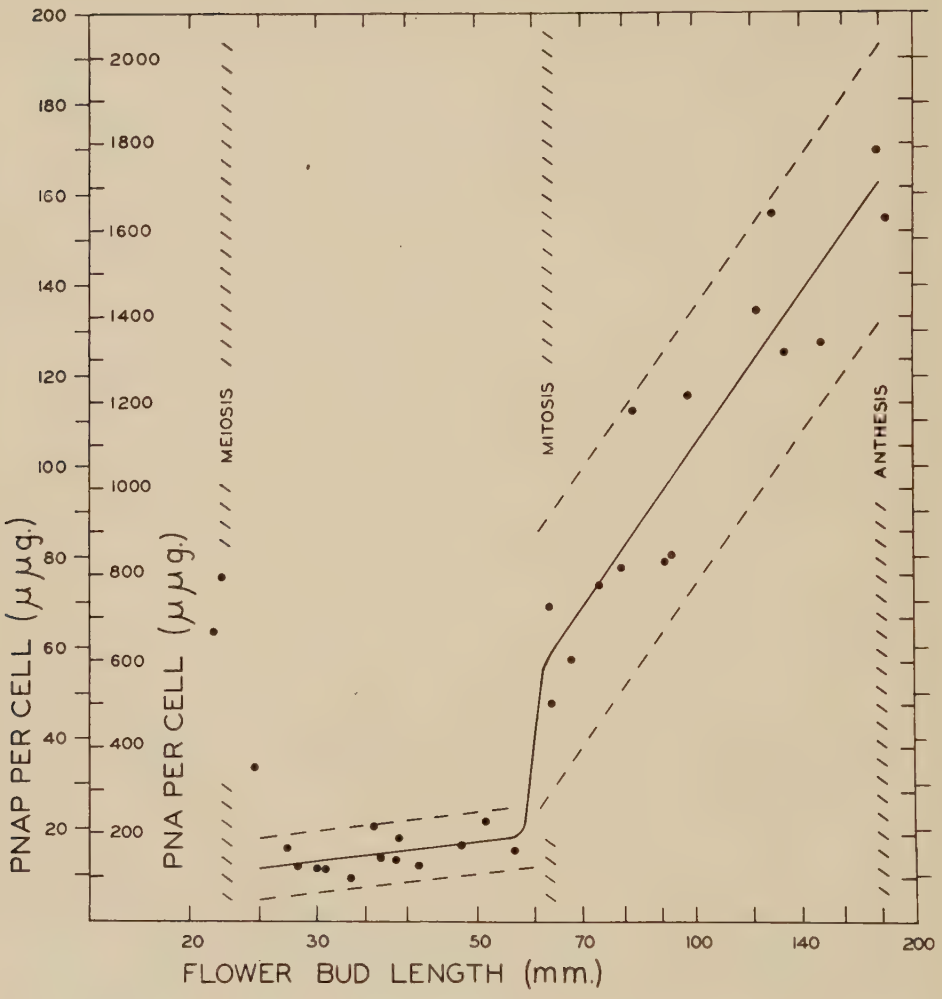

Fig. 3. PNAP and PNA content of developing microspores and pollen grains. Solid lines have been fitted by least squares. Broken lines indicate 95 per cent confidence intervals about the fitted lines.

These interphase rates of increase of DNAP, which have been calculated in terms of logarithms of bud length, may be converted into approximate rates of increase in μμg per day by multiplying each of them by the growth rate of a flower bud, 0.0765 (7). The rapid increase in DNAP at microspore mitosis appears to occur within one day. Its rate has been estimated as the difference between the DNAP values for 58 and 62 mm. bud length,

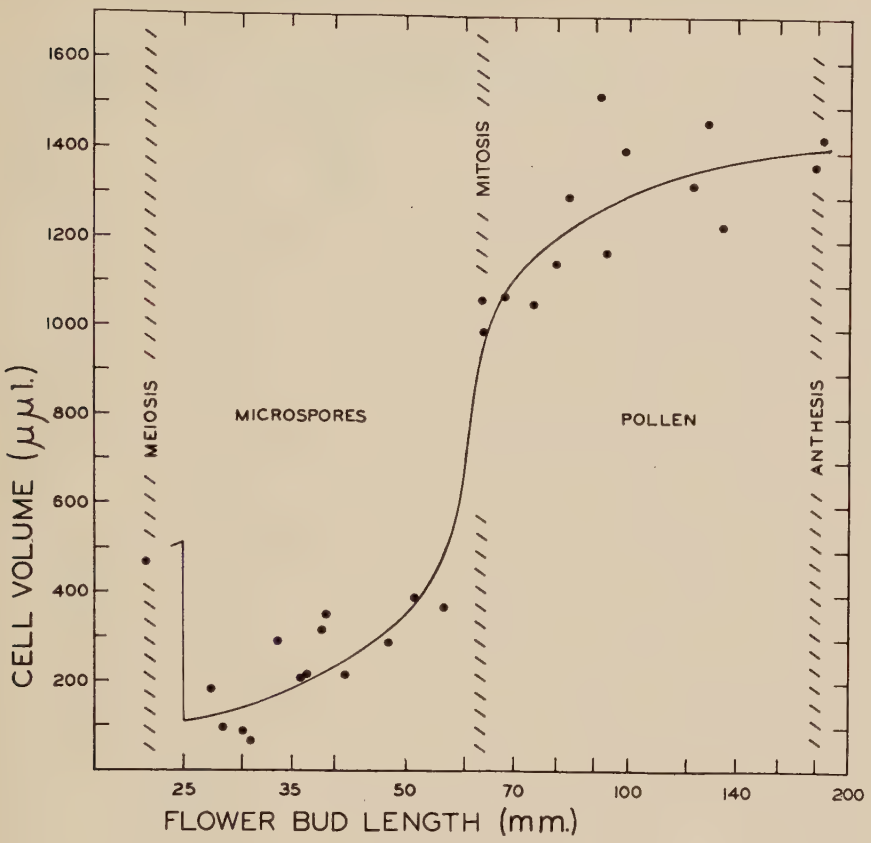

Fig. 4. Approximate volumes of developing microspores and pollen grains, calculated from hematocrit data and cell counts. Line fitted by eye.

calculated from the fitted equations. These daily rates of increase are also listed in Table II. The rate of increase during the pollen interphase, between mitosis and anthesis, is perhaps twice that during the earlier microspore interphase. The sudden rise in DNAP at mitosis, occurs at a rate which appears to be about 30 times as great as that during the microspore interphase.

Since the developing pollen grain contains two nuclei, one might divide the DNAP values obtained for pollen grains by two, to obtain the average amount of DNAP per nucleus. This has been done for the data above 60 mm. bud length, and points are plotted, together with the microspore values, in Fig. 2. A single straight line has been fitted to all these points. Its

Table II

Constants in Equations of the Type $Y' = a + b\ lnL_b$, Fitted to Nucleic Acid Content of Microspores and Pollen Grains; and Calculated Rates of Increase of Nucleic Acid Content.

Stage of Development	Bud Length (mm)	a	b	Rate of Increase ($\mu\mu$g per day)
DNAP per microspore, from meiosis to mitosis ..	25—58	—14.02	5.98	0.457
DNAP per microspore, just before mitosis	58—62	—	—	ca. 14.5
DNAP per pollen grain, from mitosis to anthesis	62—180	—22.49	11.44	0.875
DNAP per nucleus, from meiosis to anthesis	25—180	—17.87	7.11	0.544
PNAP per microspore, from meiosis to mitosis ..	25—58	—15.83	8.35	0.639
PNAP per microspore, just before mitosis	58—62	—	—	ca. 38.3
PNAP per pollen grain, from mitosis to anthesis	62—180	—363.62	99.35	7.600

slope, b, is 7.11, which indicates a constant rate of increase of DNAP of 0.544 $\mu\mu$g per nucleus per day throughout microspore and pollen development, except for the above-mentioned sudden increase at mitosis. (The fact that this rate is somewhat more than that during the microspore interphase, and also more than half that pertaining to the whole pollen grains, is explained by the fact that the rapid premitotic rise amounts to more than a two-fold increase. Dividing the later points by 2 does not entirely compensate for it.)

The equations fitted to these lines may be used to calculate expected values of DNAP at various stages of development. Dividing these values by 0.099[6] gives expected amounts of DNA. Such calculated values are given in Table III, for several critical points in development: 1. late meiosis, where diploid microsporocytes or their haploid products are present, still enclosed in the microsporocyte wall; 2. early microspore interphase, where the microsporocyte wall has broken and the young microspores have been released; 3. the end of microspore interphase, just before DNAP increases suddenly; 4. a point which might be regarded as early mitosis, just after the sudden DNAP increase; and 5. anthesis, when the pollen grains are mature. The ratios of DNAP content at each of these stages, to that of the early microspores, at 25 mm. bud length, are also given in Table III, for comparison. The value for microsporocytes in late meiosis is 4.8 times that of the early microspores. Since each microsporocyte gives rise to four equal microspores, one would expect a ratio of 4. The sudden premitotic increase from 10.2 $\mu\mu$g of DNAP at 58 mm. bud length to 24.7 $\mu\mu$g at 62 mm. is

TABLE III

Nucleic Acid Content per Microsporocyte, Microspore, and Pollen Grain at Various Stages of Development (Calculated Values).

Stage of Development	Bud Length (mm)	DNAP (μμg)	DNA (μμg)	Ratio	PNAP (μμg)	PNA (μμg)	Ratio
Late meiotic microsporocyte	21.7–24.6	25.1	253	4.8	57.3	602	5.2
Earliest microspore	25	5.2	53	1.0	11.0	116	1.0
Microspore just before mitosis...	58	10.2	103	2.0	18.1	190	1.6
Microspore in early mitosis	62	24.7	250	4.7	56.4	592	5.1
Pollen at anthesis.....	180	36.9	373	7.1	162.3	1704	14.7

an increase of 2.4 times. If this increase is thought of as indicating a chemical prelude or correlate to the morphological doubling of the chromosomes at mitosis, it perhaps represents a true ratio of 2. It is also interesting to compare the DNAP content of nuclei of the newly formed microspores and of the mature pollen. Although nuclei at both stages have the haploid number of chromosomes, nuclei at the latter stage have 3.5 times the DNAP content of the former.

The data on PNAP content of the microsporocytes, microspores and pollen grains have been analyzed in essentially the same way as the DNAP data, except that no points have been omitted in fitting the curves. The results of this analysis are shown in Fig. 3, and Tables II and III. PNAP values have been converted to values of PNA, by dividing by the factor 0.095.[6] An ordinate showing values of PNA has been drawn for Fig. 3.

Just as for DNAP, there is a linear increase in PNAP content of the microspores during the interphase between meiosis and microspore mitosis. There is about twice as much PNAP in a microspore as DNAP, and the rate of increase of PNAP is also greater. This interphase increase is followed by a further sudden increase, which is simultaneous with the rapid premitotic increase of DNAP described above. During the pollen interphase, which follows microspore mitosis, PNAP again increases, at a rapid linear rate, 8.7 times as great as that for DNAP in the same period. As a result, the mature pollen grain contains 4.4 times as much PNAP as DNAP, and 14.7 times as much PNAP as the young microspore from which it was derived.

These changes in PNAP content may be compared with growth in size of the cells. The hematocrit readings (Table I) are crude relative measures of the total volumes of cells in each sample. Dividing this volume in each

case by the number of cells in the sample, gives an approximate relative volume of a single microspore or pollen grain. These data are plotted against logarithms of bud length in Fig. 4. Comparison with Fig. 3 suggests that there is some degree of parallelism between increase in PNAP and increase in cell volume.

DISCUSSION

One of the first points which will be noticed in these data is that the DNA content per nucleus is high in comparison with values which have been reported for various vertebrates. Since *Lilium* microspore and pollen grain nuclei are haploid, these values may be compared with values such as 3.4 μμg reported by Boivin and the Vendreleys (1) for bull sperm, and 2.82 μμg for bull sperm and 3.70 μμg for toad sperm, which Mirsky and Ris (12) have found. High values for *Lilium* were perhaps to be expected since the Liliaceae generally are noted for the large size of their chromosomes. If one assumes that an undegraded DNA macromolecule has a molecular weight of 10^6, one may estimate from Avogadro's number, 6×10^{23}, that the 53×10^{-12} gm. of DNA of a just-formed microspore represents about 3×10^7 macromolecules of DNA. This is a large number by comparison with estimates of the number of genes of various higher organisms, which are usually of the order of 10^4.

Clear evidence of a linear increase in DNA-content of a nucleus during the interphase, has, to the best of the authors' knowledge, not been presented previously. Work from Boivin's and Mirsky's laboratories on nuclei isolated from various tissues of a number of animals, has led to the generalization that the DNA-content of a nucleus is a constant for the individuals of certain species, and for various tissues of an individual animal, except for the sperm, whose nuclear DNA-content is one-half that of somatic tissues. This conclusion pertains to mature organisms and tissues, not to cells during development. Brachet and other workers (2, 17) have made assays for nucleic acids in developing *Arbacia* eggs. These results have not been clearly referred to cell numbers at the various stages, and do not give evidence relating to the question being discussed here, that is, whether the DNA-content of a nucleus is constant during interphase of mitosis. Microscopic absorption methods for determining the DNA content of single nuclei (3, 14, 15, 16, 19) have not yielded any information on the constancy or lack of constancy of DNA during interphase. Aside from any questions of the validity of the measurements, any method which

depends only on microscopic observation of cells, limits one to clearly recognizable mitotic stages in judging the developmental status of the cell and affords no adequate developmental criteria during interphase.

The rapid doubling of DNA content of the *Lilium* microspores, between about 58 and 60 mm. bud length at mitosis, is perhaps not surprising. Cytologists have long spoken of an increase in the amount of chromatin in a nucleus during the early stages of mitosis. Ris (15), using microscopic absorption techniques, has presented data indicating an approximate doubling of DNA content of onion root tip nuclei from interphase to metaphase.

What is surprising in these *Lilium* data is that this rapid doubling of DNA content at mitosis, is superimposed on considerable increases during both the microspore interphase and the pollen interphase. The DNA content per nucleus increases by a factor of about 3.5 from the end of meiosis to pollen maturity. If one considers the entire cellular life cycle of *Lilium*, it is apparent that interphase increases of this magnitude cannot occur in the division of all nuclei. If it were true of a developing meristem, for instance, DNA per nucleus would increase rapidly from one cell generation to another to absurdly large amounts. At the present time, particularly since nothing is known of DNA contents of nuclei at other stages of the life cycle of *Lilium*, one can only make suggestions about the significance of these interphase increases.

The role of the pollen grain, of course, is germination on the stigma of a flower, with formation of a pollen tube, and of two haploid sperm nuclei which accomplish fertilization of the egg and the polar nuclei of the embryo sac. If one assumes that DNA is not synthesized to any appreciable extent during the rapid growth of the pollen tube, one may suppose that the great increase in DNA which occurs during pollen development serves to build up the DNA content to a high value in preparation for germination.

A second hypothesis might be that this great increase of DNA has some morphogenetic significance in the development of such a specialized structure as the pollen grain. Huskins and Steinitz (9) have shown that polyteny and polyploidy are normal concomitants of differentiation of the cells of a root tip, and have theorized that this process has some developmental importance. Schrader and Leuchtenberger's (19) data suggest that the DNA content of nuclei of various tissues of *Tradescantia* vary. They also have speculated on the developmental significance of their observation.

Two other suggestions might be made. It may be that a particular amount of DNA, *i.e.*, about 250 μμg, is requisite for the division of any nucleus of *Lilium*, whether haploid or diploid, and that the great increase in DNA

during microspore development serves to bring the level in these nuclei up to the same point as was observed in the late microsporocytes. Or, it may be that the great DNA content of these nuclei serves to some extent to counteract the inherent genetic instability of the haploid nucleus.

Whatever the full significance of these results may turn out to be, they are a beginning toward working out the quantitative relationships of nucleic acids to cell division and development.

SUMMARY

Methods are described for preparing cell suspensions of *Lilium* microsporocytes, microspores and pollen grains; for obtaining cell counts of these suspensions; and for their analysis for pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA).

The results of these analyses have been calculated to nucleic acid content in μμg per microsporocyte, microspore or pollen grain, and the results related to logarithm of flower bud length, an index of the developmental status of the cells, and of their temporal relationship to meiosis, microspore mitosis and opening of the flower.

DNA content per cell drops sharply at the end of meiosis, with the formation of four microspores from each microsporocyte. It then increases gradually during the microspore interphase between meiosis and the microspore mitosis. At microspore mitosis DNA content doubles rapidly. In the development of the resulting binucleate pollen grain, from microspore mitosis until the opening of the flower, there is a further gradual increase of DNA content. PNA content of these cells follows the same pattern up to microspore mitosis at a level about twice that of DNA, increases sharply at mitosis, and continues to increase rapidly at a rate nine times that for DNA in the maturing pollen grain.

The absolute amounts of DNA and PNA are great. At the time of anthesis the two-celled pollen grain contains about 375 μμg of DNA and 1705 μμg of PNA.

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A CORTICAL CYTOSKELETON IN EXPANDED EPITHELIUM CELLS OF SPONGE GEMMULES

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INTRODUCTION

In 1943 the senior author published some experiments on the tension in germinated and expanded gemmules of *Spongilla*, the fresh water sponge (1). Fig. 1 shows diagrammatically the organization of this organism. In the autumn a certain category of cells ("archæocytes") of the sponge fill themselves up with granules of reserve food; they then aggregate into heaps of cells about 0.2–0.8 mm in diameter, each heap surrounded by a horny capsule. In spring the cells creep out through a micropyle in the capsule and some of them attach themselves to the substrate (in nature to the skeleton of the sponge which consists of silica needles cemented together with spongin; these cells then creep along the substrate with peculiar amoeboid movements, and in this way stretch the whole mass of cells into a low conical shape. Cellular differentiation which proceeds in this system requires tension. This tension is produced by the cells which have crept out on the substratum and have formed a basal epithelium. If the little sponge is removed by pushing with a needle, it will soon contract into a sphere; and no further differentiation will take place before the sponge, by making a new basal epithelium, has created a new tension in the system. Thus the basal epithelium is of vital importance for the development and function of the sponge.

Since this phenomenon obviously has a wide bearing on morphogenesis in general, the basal epithelium of the gemmules was subject to closer analysis. The results will be presented in a number of papers, starting with the present one.

When a sponge is removed from its substratum the basal epithelium will remain behind. The mechanism of this strong adhesion between basal epithelium and substrate will be discussed in a separate paper.

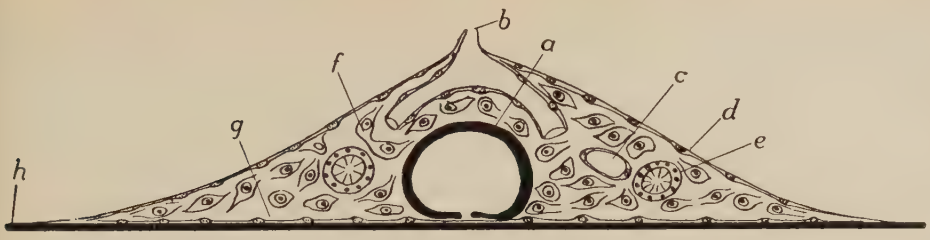

Fig. 1. Diagram of expanded gemmule. *a*, empty shell. *b*, osculum. *c*, transverse section of water canal. *d*, epithelium. *e*, ciliary chamber. *f*, archaeocytes. *g*, basal epithelium. *h*, substrate. (Brøndsted 1943.)

In a paper in 1943 (1) the senior author related the following curious observation; when a sponge, which has become spherical after removal from the substrate, is placed back onto the basal epithelium, it spread over this in 2–4 hours; if such a sponge is instead placed outside the epithelium e.g. on a clean slide, so that it has to form a new basal epithelium, its complete expansion takes 16–20 hours. From these and other observations it was clear that the cells of the basal epithelium must be able to form contact with the substratum, to *glue* themselves to it, and—once they are stretched—to develop a certain degree of rigidity.

As no cell structures in the basal epithelium were revealed by means of the light microscope which might be used for such purposes, we resorted to the electron microscope.

METHODS

The cells were investigated *without previous fixation*, as artefacts may be produced by fixation.

For examination with the electron microscope the gemmule had to germinate on an extremely thin supporting membrane which is generally used instead of slides for electron microscope work. When the gemmule had germinated, it was pushed off leaving behind the basal epithelium as a very thin coating on the membrane. This procedure is only possible if the membrane is rigidly supported. Hence the preparation was carried out as follows.

A collodion membrane is cast by placing a drop of a 2 per cent solution of collodion in amyl acetate on a water surface about 20 cm i diameter. This membrane is attached to a glass slide by holding the slide vertically over the surface and then moving it downwards into the water allowing the membrane to fold up around the edge of the slide. When the slide is removed the membrane will adhere to the glass surface. After drying the slide is ready for use.

Now the gemmule is placed on the film-side of the slide in a few drops of water. There must be sufficiently little water to ensure that it does not spread out to the edges of the membrane and force its way between the membrane and the slide.

Next the slide is placed in saturated water vapour so that evaporation from the drop during germination is avoided. When germination has reached the desired phase the gemmule is removed with a pointed tool, leaving its basal epithelium as a coat on the undamaged membrane.

Finally the membrane is washed in distilled water and allowed to dry. To enhance the contrast of the preparation in the electron microscope a thin layer of gold is applied to it by evaporation in vacuo at an incident angle of 30°.

The prepared membrane is loosened from the glass by lowering the slide into water. This will cause the membrane to float off into the water surface. The desired part can here easily be transferred to a mesh screen and is then ready for insertion into the electron microscope.

The electron microscope used was the RCA type EMU–2B installed at the Biophysical Institute (head: prof., dr. H. M. Hansen) at the University of Copenhagen.

RESULTS

It is clear that a possibly existing structure would be visible only at the edge of the cells, i.e. in places where the cytoplasm forms an exceedingly thin layer. We therefore concentrated on these parts. The cytoplasm contracts somewhat when drying in vacuo, but nevertheless some vague shades in the form of threads were seen outside the border of the cytoplasm. When we used gold shading to obtain a clearer picture a dense network was revealed everywhere outside the edge of the contracted cytoplasm. (Fig. 2, 3, 4.)

This network is composed of fibres which vary in thickness from about 150 Å to 1500 Å. A closer examination, however, show that the thicker threads are in reality complex structures consisting of several, closely placed, parallel fibres.

The gold shading was made at an angle of 30°; therefore the shadow of a particle on the screen is about three times as wide as the particle is high. Measurements of the width of the shadows indicated that height and thickness of the individual fibres are about equal.

The course of a fibre is generally a little curved, but may have fairly straight segments. The length of a fibre cannot be determined; it may exceed several microns. Only the composite fibres can be followed over some length, the small and thin ones are intervowen, into a veritable felt, so that it is impossible to determine their beginning and ending.

In several places the fibres seem to be cross-striated in a rhythmical manner, closely akin to the structure of mammalian tendon structure as revealed by the electron microscope.

In places where the receding cytoplasm forms a very thin layer it is

Fig. 2. Electron photomicrograph × 12 000.

Fig. 3. Electron photomicrograph × 12 000.

Fig. 4. Electron photomicrograph × 12 000.

Fig. 5. Electron photomicrograph × 12 000.

Fig. 6. Electron photomicrograph × 16 800.

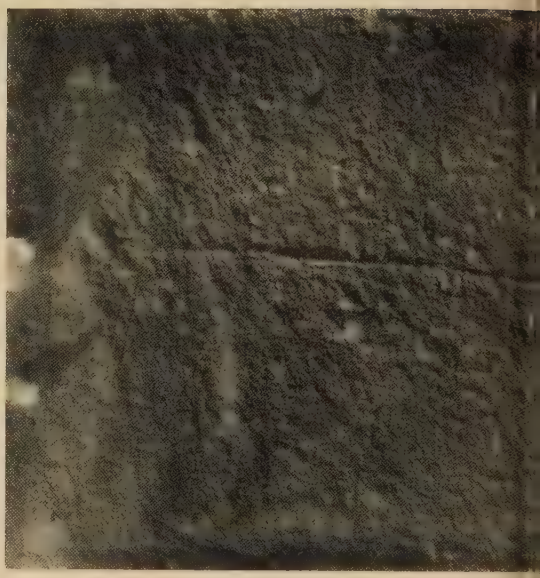

Fig. 7. Electron photomicrograph × 12 000.

possible to follow the coarser fibres for a while under this cover. This is especially obvious in Fig. 3. In such places it can also be seen that the basal network continues as a felt beneath the cytoplasm: a faint relief reveals itself, in the same manner as ropes may be seen in outline under a carpet. Fig. 5 gives the picture of a place where the goldshaded crust of dry cytoplasm has been accidentally removed during the application of the preparation to the mesh screen; it is here clearly seen that a network of fibres is lying beneath the cytoplasm. These fibres are indistinct because they are, of course, unshaded.

DISCUSSION

It is often difficult to interpret biological structures as seen in the electron microscope. Therefore the following remarks are to be considered only as a working hypothesis, but, in our opinion, a fairly sound one.

As has been described by Brøndsted (1, p. 246) the cells of the basal epithelium expand by amoeboid movements until their tendency to further expansion is balanced by the tendency of the sponge as a whole to become rounded off. This equilibrium can be maintained with a minimum of

energy expenditure by the basal membrane, because the fibres which are by now fully formed, glue the cells to the substrate and give them the required rigidity to resist the contracting force of the remaining cells.

The network of fibres is lying closely on the substratum (in this case the collodium membrane). There seems to be no cause to assume that the formation of fibres should be a specific response on the part of the cell towards some stimulus from the collodium. It is therefore presumed that the formation of fibres is a normal function of cells which have come into contact with substrates to which the cells can attach themselves.

To test the hypothesis that the fibres are formed during the outgrowth of the basal epithelium, but only reach their full development when the epithelium has attained its state of equilibrium, some investigations of the earlier stages of germination were made. Fig. 6 shows the exceedingly thin cytoplasm-film at the edge of the basal epithelium; no fibres are visible as in Fig. 5; also here in Fig. 6 parts of the crust have been removed during the making of the preparation; no fibres appear under the removed crust. At another place of the edge of the basal epithelium there are indications that fibre-formation is in progress, fig. 7.

These findings are consistent with the fact that the basal epithelium is more soft and yielding at the beginning of the germination process than later on.

Inspection of the pictures suggests that the coarser fibres are laid down first, the interstices between them being later on filled up with the dense network of younger, thinner and shorter fibres.

It may be argued that the appearance of fibres may be an artefact caused by the evaporation of water. This is not very likely. It can be seen on the pictures that the cytoplasm proper, i.e. the more fluid phase of it, dries in most places into a crust without fibre formation. In some places (Fig. 2) the cytoplasm has contracted so that some threads of cytoplasm are left on the area, formerly covered by the living cell; but these threads are quite distinct from the fibres; they show no signs of longitudinal structure, no signs of cross striation, and have a smoothly rounded form, as would be expected for dried strands of cytoplasm. It is therefore rather satisfying that the structures described above constitute a network of fibres made in and by the cortex of the cells of the basal epithelium. Such a felt of fibres will be well suited to the requirements of the basal epithelium as a rigid support for the sponge body in its attachment to its natural substrate: the skeleton.

The fact that a detached, spherical gemmule will spread out again on

the basal epithelium with a speed several times greater than if it has to make a new basal epithelium, strongly supports this interpretation of our findings.

As said before these findings may have more general bearings and methods are now being worked out to investigate the ability of cells of other suitable objects to adhere to substrates, e.g. skeletons of various forms, and to one another.

It does not seem too far-fetched to assume that this cortical skeleton has something to do with real tendon structures as well as with reticular tissues in general.

It may also be pointed out that the findings have a bearing on the well known phenomenon that tissue culture cells tend to grow out along submicroscopic structures in the substrate, see e.g. Weiss's summary of his results in 1939 (2).

These suggestions may suffice for the present; further discussions are as yet premature.

SUMMARY

Electron micrography seems to have revealed the existence of a fibrous cytoskeleton in the cortex of the basal epithelium of sponge gemmules adhering to the substrate.

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DEVELOPMENT AND USE OF THE MINOT ROTARY MICROTOME FOR THIN SECTIONING[1]

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Two widely used and well described methods of cutting thin sections for electron microscopy are those of Pease and Baker (3) in which the specimen is advanced by the lateral motion of a cone against the surface of an inclined plane, and of Newman, Borysko, and Swerdlow (4) which utilizes the thermal expansion of a previously cooled brass block. Both methods make use of the Spencer 820 rotary microtome and a steel microtome knife.

In the method to be described, highly successful routine thin sectioning is accomplished in this laboratory using a modified Minot rotary microtome[4], involving direct screw feed, and a glass knife. The precision and reliability of the technique in our hands is greatly superior to the methods mentioned above.

Description of the microtome (Figs. 1 and 2)

In the standard Minot rotary microtome, the specimen is placed in a chuck (A) on the front end of a carriage advanced by a long screw (B). The screw is turned with each revolution of the hand wheel (C) by the action of a pall (D) on a grooved "click wheel" (E). The click wheel is positively attached to the screw at the rear.

The standard microtome has been adapted for thin sectioning by changing the position of the click wheel so that a $\frac{1}{40}$ reduction worm gear can be inserted. The click wheel is mounted on the shaft of the worm (F) and the worm gear (G) is attached to the advancing screw. The finest click wheel available with the standard microtome allows, in its original position, a specimen advance of 2 microns for each tooth. With the reduction gear in place a theoretical advance of $\frac{1}{20}$ micron per tooth is

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[2] This work was done during tenure of a Fellowship in Cancer Research of the American Cancer Society, Inc., sponsored by the Committee on Growth of the National Research Council.

[3] Atomic Energy Commission Predoctoral Fellow in Biology.

[4] The microtome and adapter were manufactured by the International Equipment Company of Boston, Mass., under the direction of Mr. W. T. Smith.

obtained. Twelve settings of the pall reference block (H) permit rates of advance from $\frac{1}{20}$ up to $\frac{12}{20}$ micron.

The thin sectioning attachments may be quickly installed or removed, allowing the microtome to be used either for thin sectioning or for ordinary microtomy.

METHODS

Our technique employs the methacrylate embedding procedure of Newman *et al* (4) and the glass knife with water trough described by Latta and Hartmann (2), as adjuncts to the modified Minot microtome.

In comparing the actions of the Minot and modified Spencer microtomes, care was taken to cut sections on each from the same block of plastic. Freshly cut glass knives from the same pane of glass, similar clearance angles, and similarly adjusted trough water levels were used in both cases. Sections were examined with and without removal of the plastic embedding material. No extensive precautions were taken to eliminate vibration, friction, and play in the Spencer microtome (Hillier (1)).

RCA types EMU and EMB electron microscopes were employed. The type EMU is equipped with an intermediate lens.

RESULTS

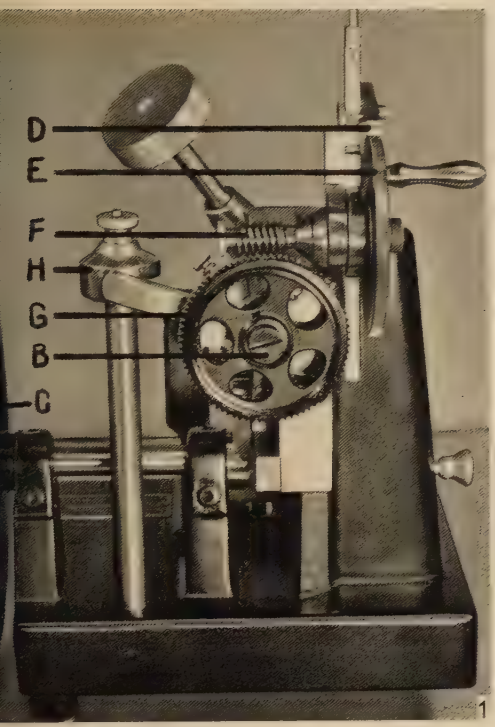
That very satisfactory thin sections are cut with the Minot microtome can be seen by examination of Figs. 3, 4, and 5. With respect to thinness, these are average samples from hundreds routinely cut in this laboratory.

Fig. 3 shows a longitudinal section through part of a squid giant nerve fiber shadowed with chromium after removal of plastic. Note the preservation of the various layers of the fiber: the outer connective tissue sheath (c), the inner more compact sheath (d), a Schwann cell (b), and the central core containing axoplasmic filaments (a).

Fig. 4 shows a portion of the cell surface of the fertilized egg of the sea urchin, *Arbacia punctulata*, with the supporting plastic left in place. The cytoplasm contains fat and yolk droplets. If the methacrylate embedding substance is to be left in place, the sections must be very thin to secure proper contrast. The use of Flemming's fixative also increases contrast because of staining by osmic acid.

Fig. 5 shows the mitotic metaphase spindle in the Arbacia egg at first cleavage, Flemming's fixation, plastic removed. The chromosomes may be seen at the metaphase plate. The fibers connecting the chromosomes to the centers on each side may be clearly seen.

Fig. 1. Close-up of thin sectioning adapter. (About one-third actual size.)
Fig. 2. Minot rotary microtome as used for thin sectioning. (About one-third actual size.)
Fig. 3. Longitudinal section of osmic-fixed squid giant nerve fiber. (About 3 700 ×.)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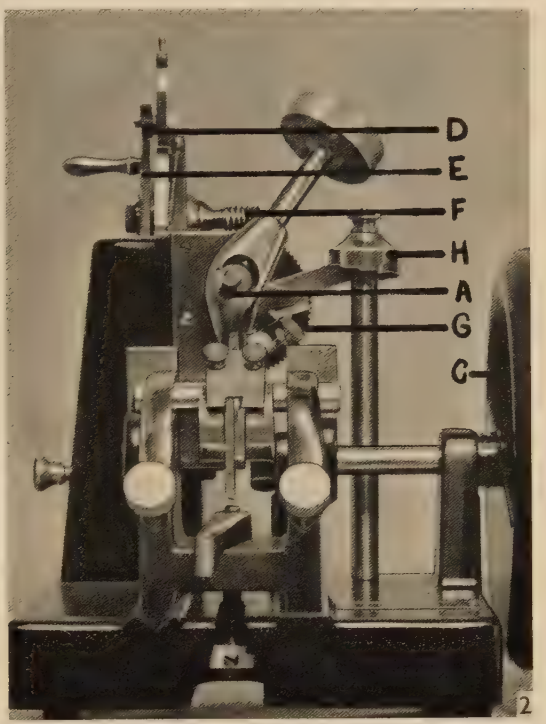
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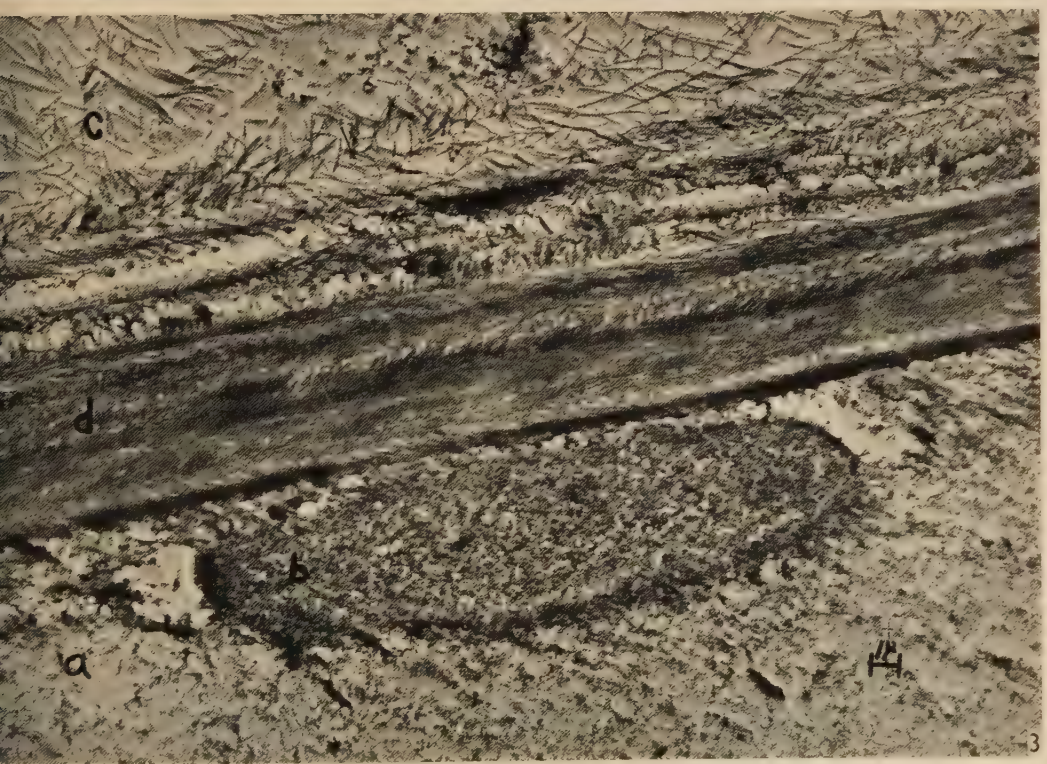
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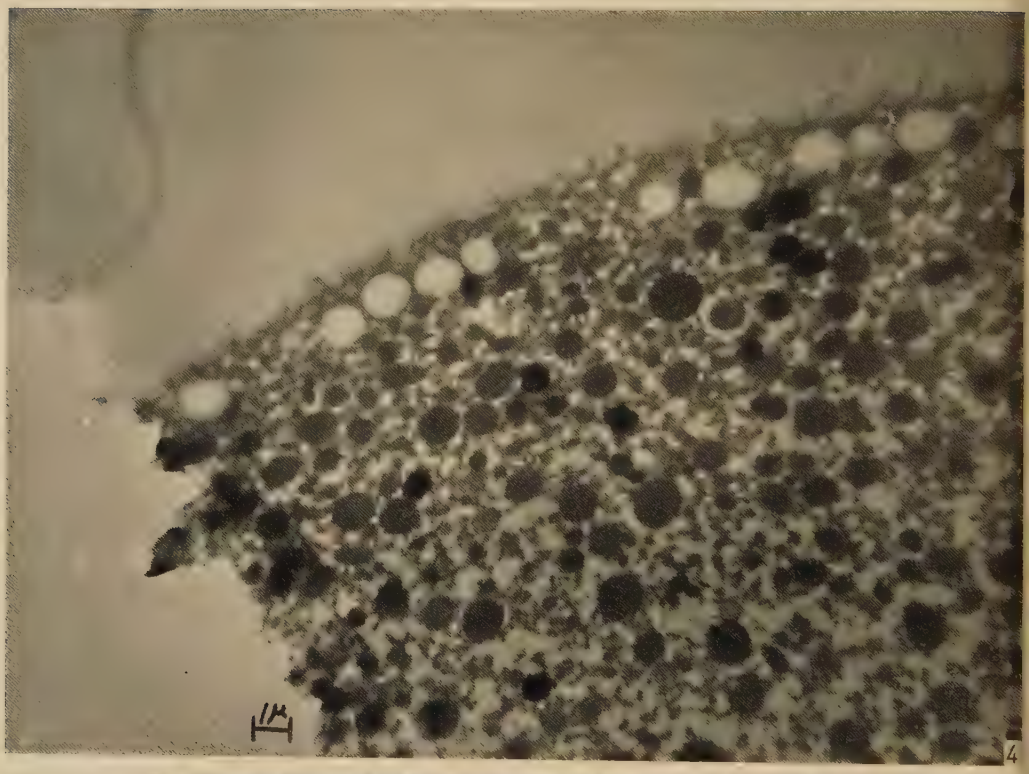

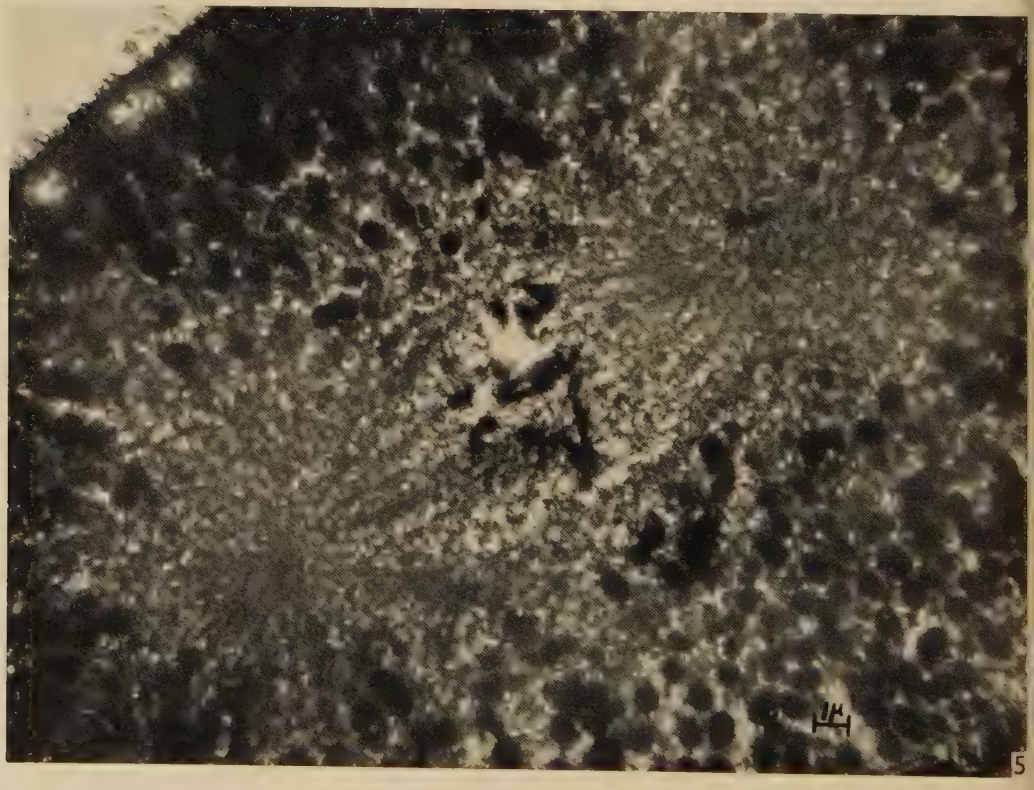

In our experience the following conditions are desirable: maximum hardness of the polymerized butyl methacrylate embedding medium; minimum specimen surface to be cut (less than 1 mm on an edge is preferable); a sharp glass knife cutting edge; minimum clearance angle between the specimen block and the cutting edge of the knife; maximum water level on the knife surface, and constant temperature of the microtome, specimen, and knife.

The greatest difficulty encountered in the cutting procedure arises from compression of the sections. Hardness of the specimen block, height of the water meniscus, and sharpness of the knife appear to be the most critical factors controlling the amount of compression.

Although one section is cut for each advance of the tooth on the click wheel, not all sections are of exactly the same thickness. Almost all, however, are suitable for electron microscopy. The greatest variation in thickness is encountered at the outset of cutting a ribbon of serial sections after the microtome has been idle for a few minutes. Of critical importance here are the factors of local temperature variations, elastic recoil of the specimen block, and compression of oil interfaces in the moving vertical ways.

The thickness of several randomly chosen sections cut with the microtome set at $\frac{1}{20}$ micron has been measured by shadowing the edges of the flattened sections with chromium and taking an appreciable number of readings along their lengths. Calculations of the thickness of the edges of various sections range from 200 to 500 A.

The variation in these measurements supports the visual observation that the thickness of the sections varies somewhat from cut to cut and from one place to another in a given section. We consider that these figures are somewhat low because of possible wedge effect at the section edge.

Preliminary measurements with multiple beam interferometry (5) using Hg yellow light indicate that other sections chosen at random measure in thickness from 400 to 800 A. The slightest compression and wrinkling of the section increases its apparent thickness by this method.

Thus it is possible to confirm that with the microtome set to advance the specimen 500 A per cut, sections of this order of thickness are indeed produced, though with some variation around the mean.

Fig. 4. Section showing cell surface of fertilized egg of *Arbacia punctulata* about 45 minutes after insemination. Plastic left in place. (4 700 × .)

Fig. 5. Section through metaphase spindle of dividing egg of *Arbacia punctulata*. Plastic removed. (4 100 × .)

DISCUSSION

The success of the adapted Minot rotary microtome is due most probably to the stability of the advancing mechanism. Essentially all backlash can be removed from the succession of tightly interlocking gears. The binding produced in the advancing sliding ways of the Pease and Baker adapted microtome is avoided by the use of direct screw feed, and the absence of a heavy thin sectioning wedge has eliminated any torque or overhang on the shaft carrying the specimen.

The advantages of glass knives over the laboriously sharpened steel knives have been apparent no matter what type of specimen advance has been employed.

The helpful advice of Dr. J. F. Hartmann and Mr. William G. Strovink concerning the adaptation of the microtome is gratefully acknowledged. This work was carried out under the general direction of Prof. F. O. Schmitt, whose constant stimulus is deeply appreciated.

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NORMAL OLIGODENDROCYTES IN TISSUE CULTURE

A PRELIMINARY REPORT ON THE PULSATILE GLIAL CELLS IN TISSUE CULTURES FROM THE CORPUS CALLOSUM OF THE NORMAL ADULT RAT BRAIN[1]

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In a study on the behavior in tissue culture of tumors of the glioma group in Man, in 1935 Canti, Bland and Russell (2) reported upon the appearances of living oligodendroglial cells. These neoplastic oligodendrocytes were described as "small polygonal cells with densely granular cytoplasm and excessively thin dichotomously branching processes which are easily protruded and withdrawn. A peculiar feature of these cells is the rhythmical pulsation that is sometimes seen."

The purpose of the present paper is to report on the behavior of similar or identical cells maintained in tissue cultures from normal adult rat brain.

Rat corpus callosum was selected as a test material for culture since the present experiments were designed eventually to explore the possibility of extending *in vitro* the work on experimental cyanide leucoencephalopathy in rats recently reported by one of us (5). Cells, which for reasons to be shown presently are presumed to be oligodendrocytes, were obtained in a high proportion of these fragments of corpus callosum. Explants were also taken from the optic chiasma, basis pedunculi and from peripheral nerve; but in the case of all these other preparations of rat neurofascicular matter no growth whatsoever has so far been obtained. In the case of adult rat cortex cerebri no cells at all resembling the pulsatile oligodendrocytes of the corpus callosum have yet been obtained, nor in the preparations of posterior

[1] Aided by a grant from the U.S.P.H.S., RG 333 (C2).

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root ganglion. Since, however, the number of such latter preparations has so far been small we are unwilling to commit ourselves as yet to any final opinion as to the potentialities for growth of other cell forms in fragments from these two sources.

In the instance of cortex cerebri it may be said that a program of researches has for some time been in progress in this laboratory, designed to describe the morphology and behavior of cells from the adult brain cortex. In addition to various animal materials, tissue obtained as biopsies during lobotomy procedures is being cultivated. While some explants are derived from subjects with a diagnosis of schizophrenia, specimens are also obtained from procedures performed on patients for the relief of intractable pain due to widespread malignant disease. Operations for the treatment of various traumatic injuries and for the removal of intracranial tumors make available additional material. Preliminary reports on this program have been published by Pomerat, Ewalt, Snodgrass and Orr (8 a and b). While no opportunity has yet arisen to attempt to grow oligodendrocytes from the corpus callosum of human brain in the same way as for rat brain we believe, however, that we have indeed seen oligodendrocytes showing characteristic rhythmical contractions in a cinematographic film made of outgrowing cells in tissue cultures from human cortex cerebri. The material was obtained in this instance during prefrontal lobotomy on a male patient, aged 40 years, operated upon for the relief of intractable pain from inoperable carcinoma bronchi with extensive metastases. The fragments of tissue supplied were entirely of grey matter.

METHOD

Young adult albino rats were killed by severing the great vessels at the root of the heart after rapid incision of the thorax without anaesthetic. The scalp and the nape of the neck were sterilized by soaking in 70 per cent alcohol. The brains were then removed aseptically into sterile Petri dishes. The hemispheres were separated by blunt dissection and the corpus callosum exposed with its anterior, hippocampal and posterior commissures. With fresh dissecting knives (to avoid contamination with grey matter as far as possible) portions of these white commissural bundles were removed and cut into fragments of suitable size for explantation. These fragments were then distributed variously into Carrel flasks, roller tubes, and as hanging-drop preparations, all in plasma clot made in the usual way with equal amounts of chicken plasma and chick embryo extract. In the case of the flasks and roller tubes the fluid phase consisted of 5 per cent chick embryo extract, 45 per cent chicken plasma and 50 per cent human ascitic fluid (from a case of carcinomatosis peritonei) and containing 1 000 units of sodium penicillin per ml.

RESULTS

In the hanging-drop preparations with no supernatant fluid phase, no cellular outgrowth was obtained from any of the explants. In the roller tube and flask preparations, about half of the explants developed an outgrowth of cells increasing in richness up to about a week and then showing increasingly frequent degenerative stigmata despite change of the nutrient fluid phase every three or four days. It is to be emphasized, however, that our experience with this particular tissue is still very limited. Further work with this material is evidently indicated, and because of the minimal interference by fibroblastic, endothelial and other cells it is hoped that this type of preparation may serve as a suitable system for the exploration of the special requirements for good growth of the more highly specialized glial cells of the white matter.

Successful preparations showed commencing outgrowth after about 36 hours and this increased until about one week when migratory activity became evidently diminished. This cellular outgrowth was never profuse. In a few preparations only was there some restricted outgrowth of cells of fibroblastic and endothelial character. Macrophages were moderately numerous and usually fairly readily identified by their general morphology, coarsely granular character, and active and characteristic amoeboid movement in the cinematographic films.

For the most part, however, the cellular outgrowth, though not dense, consists of little aggregates, three to seven or eight cells each (Figs. 1 and 2), of small polyhedral, pyramidal or nearly-spherical shape with rather short dichotomously branching processes. This tendency to aggregation into little clusters or chains is a highly characteristic feature of these cells as noted by Canti, Bland and Russell (2) in their tumor material.

With ordinary microscopic and with dark phase-contrast illumination these cells are always notably granular whatever the age of the preparation. This granularity is dense but the individual granules are of quite uniform size, distinctly smaller than the usual coarse granules of the macrophages. They show a comparatively large spherical nucleus, not infrequently laterally placed, with a prominent single nucleolus (Figs. 3 and 4). With phase contrast these cells can often be picked out at once because the cell outlines show notably bright refractility, though this is a feature which with this medium is of course by no means characteristic of this type of cell. Indeed, recently with the use of a B-lens it has been found possible to eliminate this largely as with the preparation shown in Fig. 5. When this refractility is

studied in the cinematographic films it is seen to become intensified and diminished alternately with the rhythmic pulsations which occur in these oligodendrocytes (Figs. 4 A to 4 B).

The processes of these cells are usually 3, 4 or 5 in number—very rarely more—and are usually about twice as long as the diameter of the cell body itself. It is apparently typical of these processes that, unlike neuritic and astroglial processes in living cultures, they are always straight—as if stretched—and that they usually show dichotomous branching. Such branching (Figs. 3 and 4) occurs commonly about one cell diameter, or sometimes a little less, from the root of the process. The secondary offshoots usually subtend an angle of 60 degrees or more with each other and are shorter than the primary branches; while tertiary offshoots are, again, usually shorter than the secondary branches. This dichotomous branching is, however, often incomplete and may be represented simply by a short flat ribbon-like expansion of rectangular or triangular shape at the extremity of the primary branches (Fig. 5). When secondary and tertiary branches are present, their terminations are similarly expanded. In the cinematograph film these ribbon-like expansions at the tips of such processes are seen to be extremely thin and highly active undulating membranes. These appear in the film (representing a 120-times increase in the actual speed of the cell movements) to dart to and fro and to right and left as the process lengthens and its tip advances. These expansions often appear to adhere to the plasma clot or to the surface of the glass, and during the contraction of the cell body they seem to anchor the tips of the processes peripherally so that the contraction of the cell body is made possible only, in these instances, by the stretching of the proximal part of the process. When the terminal expansions of the processes of neighbouring cells of the same character come into contact—and in the film this gives a strong impression that this is the result of some tropism rather than a mere fortuitous encounter—they fuse rapidly into a single fibre so that the cells become moored together by a single sharply-stretched cord at the ends of which the opponent cell bodies appear to engage in tug-of-war movements which are really remarkable to observe in the cinematographic film. In this way one cell may be tugged along behind its opponent for a

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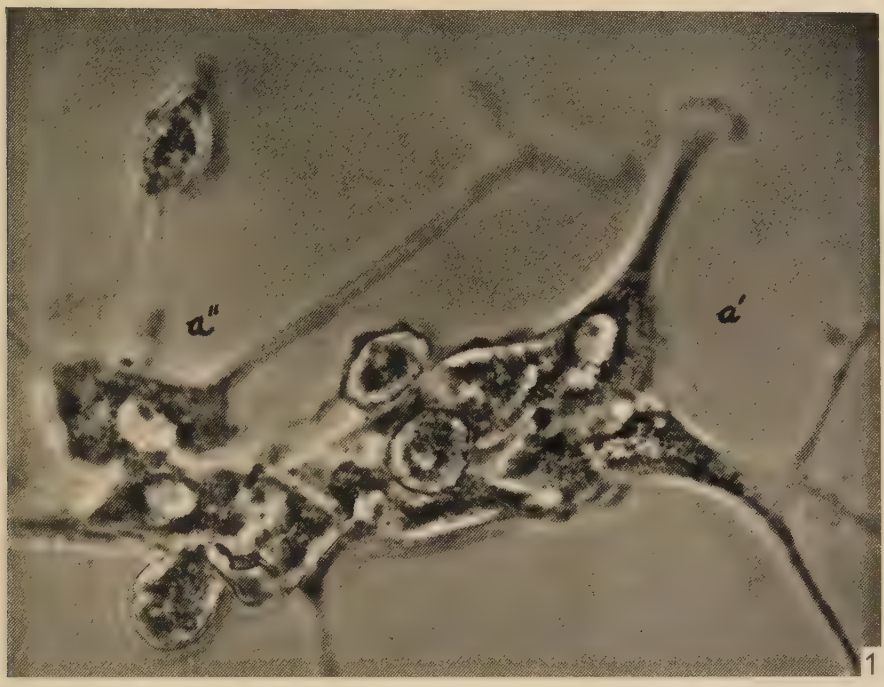
Fig. 1. Cluster of cells just beyond margin of explant of a fragment of rat corpus callosum cerebri. Note the prominent nucleolus, the uniform granularity of the cytoplasm and the long dichotomously-branched or spatula-like tipped processes of the cells a′ and a″. Dark phase-contrast × 600.

Fig. 2. Cluster of cells in outgrowth. Preparation fixed in formalin and stained by Jacobson's method. Note the cluster of cells numbered "1" to "6" with the features of oligodendrocytes described in text. × 500.



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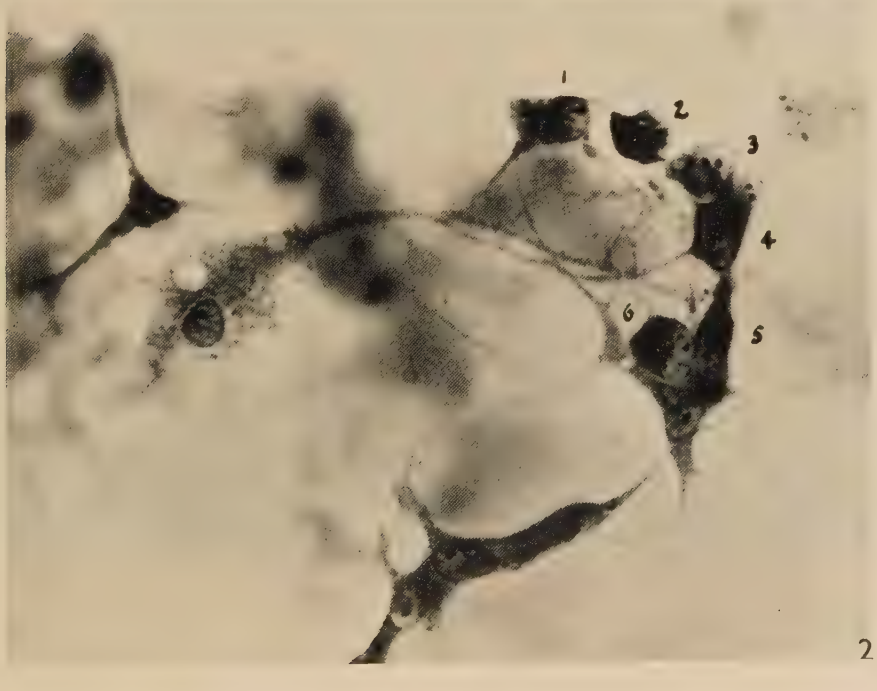

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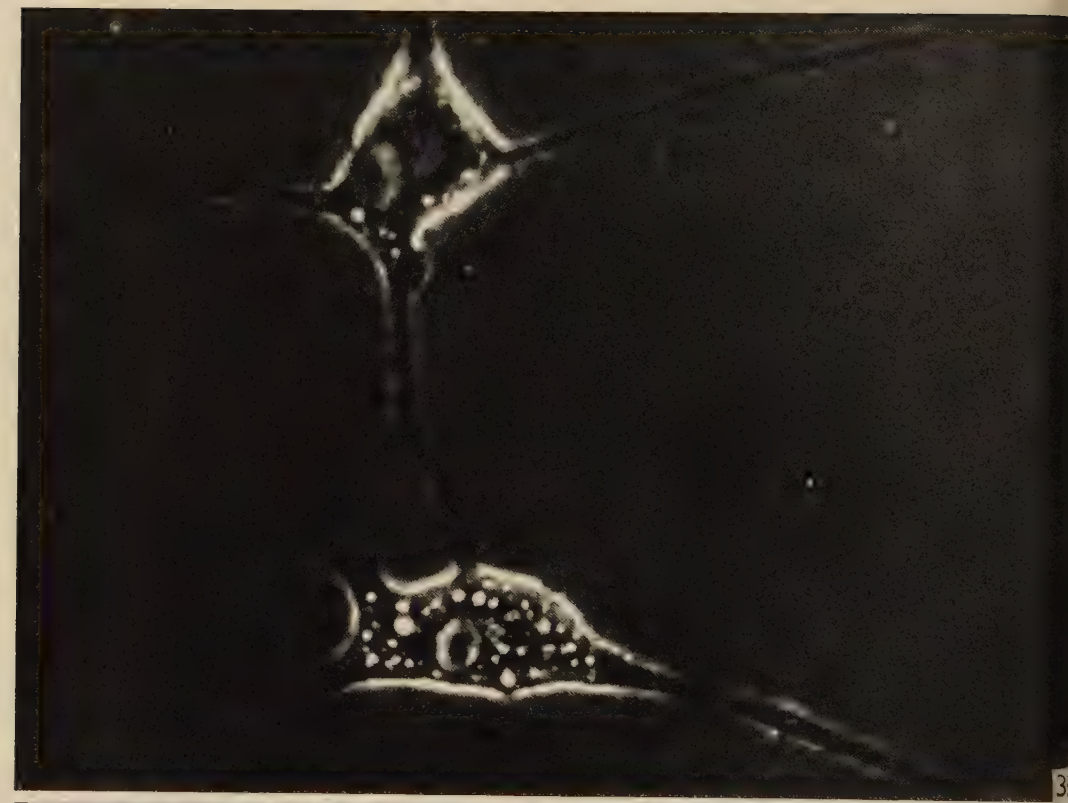

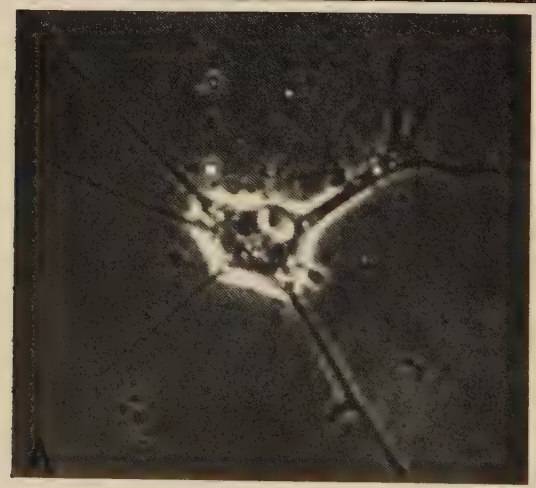
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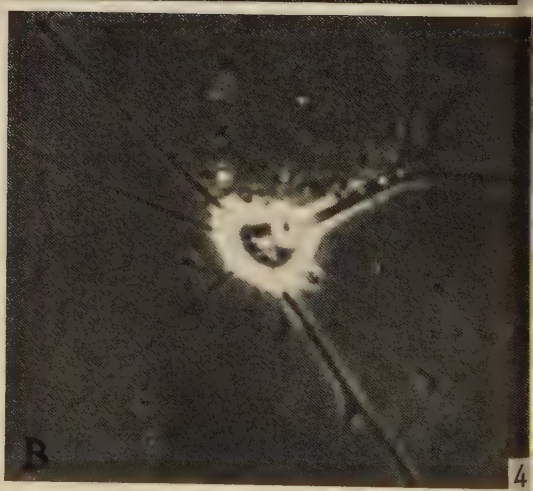
B

Fig. 5. Detail of cell processes.
Dark phase-contrast × 600.

short distance and then the process may be reversed and the opponent is pulled back. Whole clusters of four or five or more cells of this type can become chained together by these processes, each pursuing its own and apparently independent rhythm as the group sways jerkily in this direction and then in the opposite direction. This tug-of-war between pairs or groups of cells may continue uninterruptedly for hours but sometimes the connecting

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Fig. 3. Still enlargement taken from selected frame of moving film. These two oligodendrocytes showed strong rhythmical contractions. The branched processes are shown and also the fusion between certain processes of these two adjacent cells.
Dark phase-contrast × 600.

Fig. 4. Normal oligodendrocytes in a culture from a fragment of rat corpus callosum. "A" shows the expanded phase and "B" the contracted phase of the same contraction cycle. Reproduced from a cinematographic film. The time-interval between "A" and "B" was 2 minutes exactly.
Dark phase-contrast × 400.

fibres snap and the ends are withdrawn suddenly into the parent cell bodies. Whether this is an effect of the strong, though intermittent, lighting used in the time-lapse photography is not yet evident but, generally, it seems to be a feature of the oligodendrocytes that their protoplasmic processes can be readily extruded and equally readily withdrawn without any apparent impediment to the active contractility of the cell bodies.

These contractions or pulsations, so characteristic of the cell are remarkably rhythmical and they appear as powerful movements within the cell body itself. By direct comparison of the cinematograph films of our own material with the film made in 1935 by Canti, Bland and Russell (see footnote[1]) it is evident that the rate of the movements of pulsation of the normal oligodendrocytes from rat corpus callosum is approximately the same as of those of the oligodendrocytes obtained from human tumor material by those workers. Using direct timing with a stopwatch from the moving film and making the necessary calculations from the data on the photographic exposure intervals and the speed of the film, supplied with the original film of these authors, we estimate that in the case of Canti, Bland and Russell's material the rate of contraction of these cells was in the region of once every five minutes. By the same timing method with films from our own material the rate of pulsation of the normal rat oligodendrocyte was also found to be about five minutes for each contraction cycle. Furthermore, with our own material, recourse has been had to reproduction in still photographs of a series of 96 frames from the ciné-film under strictly constant conditions of enlargement and exposure time. Then by measuring the actual areas covered by the outlines of the cell bodies in the series of prints, it has been possible to find exactly the maximal and minimal areas of the same cell over a period of two complete contractions. These areas plotted graphically provided a simple and reasonably accurate method for the present purpose of analysing the different phases of the contraction. By this method, it was found that each whole contraction cycle took four and one-half minutes. It has not, of course, been possible to analyse in this way every contraction cycle during the time covered by the whole film but ordinary scrutiny of the moving film shows that the rate is constant for each cell and that all oligodendrocytes in these preparations beat at about the same rate. Further, the contraction, as seen in the moving film, is similar to that of a muscle cell, though very much slower, and consists of a more protracted expansion ("diastole-like") phase and a more rapid ("systole-like") contraction phase. These two phases are accordingly reflected in the graph of the contraction curve. The expansion

[1] A copy of this film was supplied by courtesy of the British Imperial Cancer Campaign.

phase takes approximately 3 minutes and the contraction phase one and one-half minutes approximately. Since this latter phase occupies only one and a half minutes, the change in shape and volume is just sufficiently rapid to be appreciated even by direct observation of the cell through the microscope. There is visible shrinkage of the cell body, and a loss of convexity with sharp increase in refringence of the contours of the cell body under dark phase-contrast illumination.

Direct comparison has been made of the movements of these oligodendrocytes with those of other cells in moving films made in this laboratory on growing cultures not only of brain but of other tissues. We believe it is impossible to mistake the movements of outgrowing fibroblasts, smooth muscle cells, endothelial cells and mesenchymal elements generally for these rhythmical contractions of oligodendrocytes. Likewise, it can be affirmed that the movements of astrocytes in tissue culture are much slower and of a different—rather gliding—character as was clearly shown also in the film on the behavior of gliomata in tissue culture made by Canti, Bland and Russell (2). On the other hand the movements of macrophages which occur in the cultures of rat corpus callosum, as in all our cultures from other sources, are of an altogether different nature being much more rapid and of an amoeboid locomotory character. The cytoplasmic movements in macrophages are displacement alterations of irregular flowing character and not rhythmically contractile or pulsatile movements like those of the oligodendrocytes. As for microgliocytes, there is amongst the uncertainly identified elements of the outgrowths in these cultures of rat corpus callosum a small number of cells of rather coarsely granular character not unlike macrophages but lacking their great motility. These are usually somewhat rod-shaped and exhibit a tendency to throw out numerous little globular or bud-like pseudopodia. They bear morphological resemblance to those reported by Costero (3) in 1930 and we regard these as microgliocytes. Further study of these interesting cells is envisaged. Meanwhile it can be said that they are devoid of the rhythmical pulsatility which we believe to be characteristic of the oligodendrocyte.

DISCUSSION

Reasons are given in the foregoing analysis of results for the view that the cells in tissue cultures of rat corpus callosum which are capable of rhythmical beating or contraction are oligodendroglial. They present, particularly for tissue cultures, a considerably uniform morphology rendering it possible to identify them with reasonable accuracy even on ordinary inspection of

the preparation under the microscope or in still photomicrographs made therefrom without necessary recourse to the moving film. Further, their morphology in the living state is similar to that of fixed and stained preparations from normal uncultured brain material, apart from the fact that in fixed and stained material the processes of oligodendrocytes tend to be wavy. The living cells from the rat brain shown in the illustrations of the present paper are strikingly similar to some of those illustrated in del Rio Hortega's classical papers of 1921 and 1928 (see 4 a and b) —e.g. those in Fig. 5 and 6 of his 1921 paper and Fig. 9 and 17 of the 1928 paper. Indeed it seems from Hortega's illustrations that the variety of morphological forms of the oligodendroglia is even more widely varied in the normal state in the tissue of the living animal than in culture, and that the cells which are seen in cultures are those of a rather simpler variety—those which, following Hortega's 1928 paper, might be classifiable as the "Type I of Robertson"—while the more complex giant forms with long spiral ribbon-like processes have not yet been seen in culture. While undulating filamentous tips are commonly seen on the advancing processes of growing cells of many types other than oligodendrocytes in tissue cultures, it is tempting to speculate that these curious rectangular and triangular thin plate-like terminals of the oligodendrocytic processes in tissue culture are the precursors of the more complex patterns of oligodendrocytic process that entwine, sometimes almost bandage-wise, the myelinated nerve fibres.

While it seems fairly certain that oligodendrocytes are the only cells within the central nervous system showing these rhythmic pulsations or contractions it is not yet known whether all oligodendroglial cells in culture—and, by inference, in the normal living state—show this activity. Canti, Bland and Russell (2) state that it was "sometimes" seen. Such, it is felt at present, is our experience; for in four cinematographic films made from different preparations not all cells seen with the apparently genuine morphological features of oligodendrocytes show fully-declared pulsatility though most show rather subdued movements of a similar character. But for the moment it seems probable that pulsatility is a general characteristic of normal adult oligodendrocytes, and of these cells only in brain, and that is an intermittent process with active and resting phases.

As to the significance of this muscle cell-like activity of the oligodendrocyte we can only speculate at the moment; but with this most numerous of the brain cells and about whose function we know next to nothing except that it may be associated with the metabolism of myelin, the fact of its pulsatility may be of fundamental importance. It is interesting perhaps that in our

present cultures there has been no neuritic outgrowth whatsoever so that the outgrowth of oligodendrocytes is apparently in no way dependent upon the growth of nerve fibres. Nor, it is interesting to note, have we noted so far any sign of myelin deposition in any of this culture material from adult rat brain though we have been on the look-out for this with much interest since hearing the recent report from Dr. Margaret Murray's laboratory (7) on the formation of myelin in chick embryo ganglion cultures, and although we have allowed a few of our later preparations to run for 30 days.

Oligodendrocytes were found around the edges of the explants in a fairly high proportion of the present preparations, and in reasonably large numbers predominating over other cell types. It seems, therefore, justifiable to hope that with practice and with some technical improvements, as for instance in respect of nutrient media, it may be possible to provide an easily made and easily handled experimental system upon which *in vivo* biochemical studies (respiratometric and enzymatic) and investigations of the action of certain poisons can be made directly. It might be, for instance, of great interest to determine the action of minute doses of lead salts. This element, as the researches of Reznikoff and Aub showed (9) has a marked affinity for lactic acid formed in contracting muscle cells, leading to the formation within them of insoluble lead phosphate. It might, for instance, prove that lead may have a similar affinity for the contractile oligodendrocyte. If this were so we might have an immediate explanation for the role of lead in initiating some cases of demyelinating disease as there is some evidence to believe it may do in multiple sclerosis (1) and in the exogenous form of Schilder's disease (10).

It will, too, be of great interest to elucidate the normal physiological significance of this contractility or pulsatility. It seems within the bounds of possibility that these contractile cells may exert some sort of massage effect and thereby may influence the local movements and transport of tissue juices and nutrients generally, or even—more specifically—of axoplasm and myelin.

Finally, as a further line of possibly fruitful research that may be immediately followed, it should be fairly readily possible to take electrical recordings from these contractile oligodendrocytes as has been done by Olivo and Posteli (6) from contracting heart muscle cells in tissue culture. It may be again repeated that oligodendrocytes are probably the most numerous of all cells within the normal adult human brain. Whether, therefore, these contracting or pulsating oligodendrocytes may generate electric potentials which, summated, may be sufficient to contribute to the electroencephalographic complex is a fascinating problem in itself. In this connection, the fact that these cells show such a characteristic tendency to clump themselves

together, to join their processes and engage in the curious tug-of-war activity which Canti and his collaborators first observed and which we have just described, raises a very real possibility that groups, at least, of these cells may from time to time beat synchronously and evoke a summated discharge which might be responsible for some episodic peak in the complex blur of electrical activity of the brain.

SUMMARY

1. Cells believed to be oligodendrocytes have been obtained in outgrowths from explants *in vitro* of normal adult rat corpus callosum.

   These cells show a characteristic rhythmic pulsatility apparently identical with that described in 1935 by Canti, Bland and Russell in the case of oligodendrocytes obtained in culture from oligodendroglioma of the human brain.

   We believe also that we have seen similar cells in a few tissue culture preparations from the cortex cerebri of the normal adult human brain.
2. The morphology of this cell in the living state in culture has been described and some analysis has been attempted of its pulsation as studied by time-lapse ciné-photomicrography.
3. Some possible physiological and pathological implications of this pulsatile activity of oligodendrocytes are discussed.

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STUDIES ON THE CYCLOPHORASE SYSTEM XV THE MALIC OXIDASE[1]

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INTRODUCTION

Previous studies (7) have shown that the oxidation of all the intermediates in the citric acid cycle is implemented by the cyclophorase complex of enzymes localized within the mitochondrial units (9) from cell-free preparations of animal tissues. The ability of these many oxidases to operate without being supplemented by soluble cofactors and coenzymes[3], such as cytochrome *c* or the pyridinenucleotides[4]—in marked contrast to the behavior of the same enzymes in a more purified state—led to a further investigation of these oxidases. It was found (11, 12) that many of the oxidases in the cyclophorase complex are indeed pyridinoproteins and that, owing to the localization of pyridinenucleotides on or within the mitochondria, the various enzymes are saturated in this respect and no stimulation results when additional coenzymes are added. By various physical and chemical treatments the pyridinenucleotides could be removed, and by this technique the oxidases could then be demonstrated to be pyridinoproteins since, in this deficient condition, they would not function until pyridinenucleotide had been added back to the system. In correlation with the above results, Harman (9, 10) has observed that any of the physical or chemical treatments which cause the release of pyridinenucleotide from the cyclophorase complex also produce concomitant changes in the structure of the mitochondrial unit. Whether the pyridine-

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[1] Presented at the 116th Meeting of the American Chemical Society, Atlantic City, September, 1949.

[2] Williams-Waterman Fellow in Enzyme Chemistry, 1948–1949.

[3] Similar systems which function in the absence of added cofactors have been described in homogenates (18, 16), washed particulates (13) and isolated mitochondria (14).

[4] Diphosphopyridinenucleotide (DPN) and triphosphopyridinenucleotide (TPN).

nucleotide is free and diffusible, but confined within the mitochondrion by a membrane-like barrier (4), or rigidly bound to the surface of a mitochondrial macromolecule (10), is still an unresolved question. However, regardless of the interpretation the evidence cited above (11, 12) clearly implies that the pyridinoproteins in the cyclophorase complex can exist in two states, *viz.* (a) not requiring, and (b) requiring added pyridinenucleotide. It is the purpose of the present paper to compare the properties of one pyridinoprotein (*i.e.*, the malic oxidase) in these two states which will be referred to, hereafter, as (a) *conjugated*, and (b) *dissociated*. These specific terms are employed chiefly as a convenience and are intended only to describe the general relationship of the pyridinenucleotide to the apo-oxidase.

EXPERIMENTAL

Demonstration of a Conjugated Malic Oxidase in the Cyclophorase Complex. As shown in Table I the malic oxidase in a freshly-prepared cyclophorase gel requires no pyridinenucleotide and is actually inhibited by added coenzyme. However, unless certain precautions are taken some dissociation of the coenzyme occurs even in the freshly-prepared gel. Thus, by omitting fluoride which inhibits enzymatic cleavage or by incubating the enzyme anaerobically at 38° before permitting the oxidation process to start a significant amount of dissociation is demonstrable.

Dissociating Procedures. Aging at 0° for a period of 1 to 3 days, incubation at 38° for periods up to ten minutes, repeated freezing and thawing, washing with deionized water, precipitation by acetone, exposure to high salt concentrations, capryl alcohol, arsenite, gramicidin or 2, 4-dinitrophenol all lead to a dissociated malic oxidase. The reader is referred to Table IV of the preceding paper (12) for a tabulation of the percentage dissociation induced in the malic oxidase by the procedures mentioned above. Some of these treatments, such as exposure to arsenite, merely dissociate the coenzyme but leave the malic apo-oxidase still attached to the particles. Other procedures, such as exposure to capryl alcohol, effect a visible change in the gel and lead to the release of some soluble protein; but, again, most of the malic apo-oxidase remains on the particles. Finally, procedures like precipitation with acetone result in an extensive breakdown of the original, organized protein complex and a portion of the malic oxidase becomes soluble. However, in this paper all comparisons have been made between the conjugated and dissociated oxidases, each existing on the particles.

TABLE I

Demonstration of a Conjugated Malic Oxidase.

Incubation	Conditions: Reaction temperature	Reaction velocity sec.$^{-1}$ × 10^3: With DPN	Without DPN	Disssociation per cent
1. 2 min.	38°	27.0	23.2	14.1
2. None	38°	21.7	20.4	6.0
3. None	20°	4.2	5.0	—19.8

Each Thunberg tube contained 1.0 cc of R_3K[1], 0.3 cc of 0.01 M adenylic acid, 0.2 cc of 0.02 M magnesium chloride, 1.0 cc of 0.1 M phosphate buffer (pH 7.5), 0.5 cc of 0.1 per cent 2,6-dichlorophenolindophenol (sidearm), 500 μgm of DPN where specified, 0.5 cc of 0.1 M DL-malate, 1.0 cc of 0.33 M fluoride (in experiments 2 and 3) and water to make 5.5 cc.

A blank in each of the 3 experiments, consisting of DPN but no malate, gave a negligible contribution.

Percentage dissociation is defined in terms of reaction velocities (the reciprocal of the dye reduction time) as the ratio:

$$\frac{(\text{With DPN} - \text{Without DPN})}{(\text{With DPN})} \times 100$$

TABLE II

Effect of Inhibitors.

Inhibitor	Conjugated: Velocity sec.$^{-1}$ × 10^3	Conjugated: Inhibition per cent	Dissociated: Velocity sec.$^{-1}$ × 10^3	Dissociated: Inhibition per cent
None	25.0	0	28.6	0
Capryl alcohol	5.1	80	30.3	—6.0
Arsenite	1.7	93	28.6	0
2,4-Dinitrophenol	1.7	93	28.6	0
Gramicidin	1.7	93	28.6	0

For the conjugated experiments each Thunberg tube contained 1.0 cc of R_3L, 1.0 cc of 0.1 M phosphate buffer (pH 7.5), 0.3 cc of 0.1 per cent 2,6-dichlorophenolindophenol (sidearm), 0.5 cc of 0.1 M DL-malate, 0.3 cc of 0.01 M adenylic acid, 0.2 cc of 0.02 M magnesium chloride, inhibitor as described below and water to make 3.3 cc.

For the dissociated experiments each Thunberg tube contained 0.5 cc of an R_3L acetone powder (10 per cent by weight in 0.9 per cent potassium chloride), 0.5 cc of 0.1 per cent 2,6-dichlophenolindophenol (sidearm), 0.2 cc of 1.0 M DL-malate, 2.0 cc of 0.1 M dimethylglycine buffer (pH 9.5), 500 μgm of DPN, inhibitor as described below, and water to make 4.2 cc.

The amounts of inhibitor were: .03 cc of capryl alcohol, 0.2 cc of 0.1 M arsenite, 0.2 cc of 0.1 M 2,4-dinitrophenol, .03 cc of gramicidin (1 per cent in ethanol).

Each tube was incubated at 38° for 2 minutes before tipping.

Percentage inhibition is defined in terms of reaction velocities as the ratio:

$$\frac{(\text{None} - \text{Inhibitor})}{(\text{None})} \times 100.$$

[1] R_3K and R_3L indicate a thrice-washed cyclophorase residue of rabbit kidney and liver, respectively.

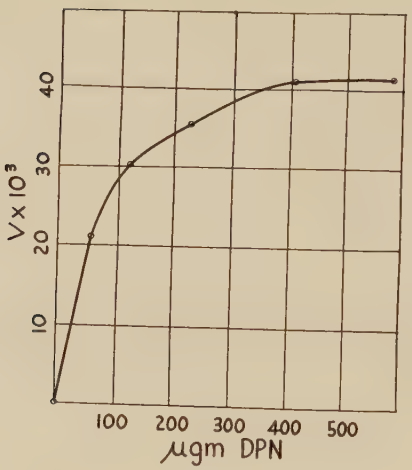

Fig. 1. Each Thunberg tube contained 0.5 cc of R_3L (previously incubated for 5 minutes at 38° in the presence of 0.1 M arsenite followed by two washings in 0.9 per cent potassium chloride), 0.2 cc of 1.0 M DL-malate, 2.0 cc of 0.2 M 2,2-dimethyl-1, 3-propanediol buffer (pH 9.5), 0.5 cc of 0.1 per cent 2,6-dichlorophenolindophenol (sidearm), DPN as indicated (sidearm) and water to make 4.2 cc. Each tube was incubated at 38° for 2 minutes before tipping.

Transforming Agents and Inhibitors. The oxidation of malate in the cyclophorase system is abolished by capryl alcohol, arsenite, gramicidin and 2,4-dinitrophenol. Hence, these reagents could be classified as inhibitors of the cyclophorase malic oxidase. But since these reagents have been shown to transform the conjugated cyclophorase oxidases into the dissociated state (11), it would be more correct to consider them as "transforming agents" rather than inhibitors, since the inhibition can be removed merely by supplying coenzyme at an adequate level to restore the activity of the dissociated oxidase. After transformation has taken place (*e.g.*, by acetone treatment) dissociated oxidase in presence of excess of coenzyme is completely insensitive to these "inhibitors" as illustrated in Table II.

Requirement of the Split Oxidase for Pyridinenucleotide. Figure I shows the level of DPN necessary to reactivate the dissociated malic oxidase prepared by exposure to arsenite at 38°. The saturation and half-saturation values in this experiment are about 400 and 50 μgm of DPN per cc. However, owing to concomitant destruction of DPN by the tissue preparation (11, 12) the actual saturation values are somewhat lower.

Effect of pH Upon Activity. The conjugated malic oxidase functions optimally in the pH region of 7.0–8.0. If exposed to either acid or alkaline

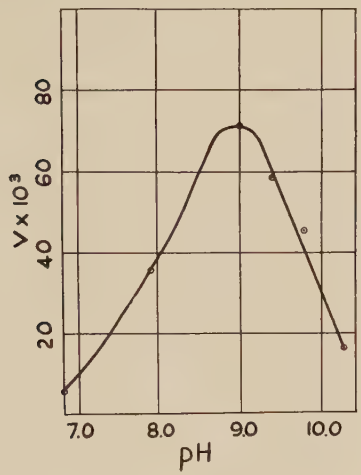

Fig. 2. Each Thunberg tube contained 0.5 cc of R_3L (previously incubated 5 minutes at 38° in the presence of 0.1 M arsenite and then washed twice with 0.9 per cent potassium chloride), 1.0 cc of 0.2 M buffer pH 9.5, 0.5 cc of 0.1 per cent 2,6-dichlorophenolindophenol (sidearm), 0.1 cc of 2.0 M cyanide, 0.1 cc of 1.0 DL-malate, 500 μgm of DPN and 0.04 cc of capryl alcohol.

conditions beyond this limited range, partial dissociation occurs and one is no longer dealing exclusively with the conjugated oxidase. The dissociated oxidase, in the presence of cyanide to bind the oxalacetate, exhibits its activity over a broad range in the alkaline regions with a maximum near pH 9.0. The data illustrating this effect, shown in Figure 2, were obtained by measuring reduction times in Thunberg tubes each of which was identical with respect to contents and different only in the pH of the buffer. The actual pH of each sample was measured with a glass electrode at the conclusion of the experiment.

Inhibition by Oxalacetate. Previous investigators have observed that the malic oxidase is strongly inhibited by oxalacetate, the product of oxidation, and that the system could be studied only if the oxalacetate were removed by trapping with cyanide (8) or by transamination with glutamate (2).

The dissociated malic oxidase has a very low activity at pH 7.5, when tested in the following anaerobic system with 2,6-dichlorophenolindophenol or methylene blue as the dye:

$$\text{malate} + \text{dye} \rightarrow \text{oxalacetate} + \text{leucodye} \quad (1).$$

The amount of dye is such that during its reduction less than 1 μmole of oxalacetate is formed, yet this quantity is sufficient to almost completely

TABLE III

Effect of Cyanide Upon the Dissociated Malic Oxidase.

Additions	Velocity ($sec.^{-1} \times 10^3$)
None	14.8
Cyanide	100.0

Each Thunberg tube contained 0.5 cc of the pig heart preparation (*cf.* experimental section, reference (7) for details), 0.5 cc of 0.1 per cent 2,6-dichlorophenolindophenol (sidearm), 0.2 cc of 1.0 M DL-malate, 2.0 cc of 0.1 M phosphate (pH 7.5), 250 μgm of DPN, 0.04 cc of capryl alcohol, 0.2 cc of 2.0 M cyanide (pH 7.5) (where indicated), and water to make 4.0 cc.

TABLE IV

Inhibition by Oxalacetate.

Conjugated (at pH 7.5)			Dissociated (at pH 9.5)		
Oxalacetate added μgm.	Velocity $sec.^{-1} \times 10^3$	Inhibition per cent	Oxalacetate added μgm.	Velocity $sec.^{-1} \times 10^3$	Inhibition per cent
0	11.5	—	0	38.5	—
10	12.3	—7.0	5	22.2	42.3
40	12.1	—5.2	30	8.1	79.0
70	9.2	20.6	100	3.4	91.0

In the conjugated experiments each Thunberg tube contained 1.0 cc of R_3L, 0.4 cc of 0.1 M DL-malate, 0.1 cc of 0.1 M of phosphate buffer (pH 7.5), 0.2 cc of 0.1 per cent methylene blue (sidearm), oxalacetate as specified and water to make 3.7 cc. Each tube incubated at 38° for 2 minutes before tipping.

A blank containing 70 μmoles of oxalacetate and no malate had an infinite reduction time (zero velocity).

In the dissociated experiments each Thunberg tube contained 1.0 cc of R_3L, 0.2 cc of 0.1 M arsenite, .03 cc of capryl alcohol, 0.2 cc of 1.0 M DL-malate, 1.0 cc of 0.1 M dimethylglycine buffer (pH 9.5), 500 μgm of DPN, 0.5 cc of 0.1 per cent methylene blue (sidearm), and water to wake 4.9 cc. Each tube was incubated at 38° for 5 minutes, before tipping.

TABLE V

Recovery of Oxalacetate Incubated with Cyclophorase.

μ moles of Oxalacetate	
Initial	Recovered
1.00	0.90
10.0	9.00
40.0	40.7

The specified amounts of oxalacetate were incubated with 0.1 cc of 0.1 M phosphate (pH 7.5) and 1.0 cc of R_3L under anaerobic conditions for 5 minutes at 38°. 2.0 cc of 50 per cent trichloracetic acid was added to each tube and after filtration a 2.0 cc aliquot was withdrawn and the keto-acid determined by the spectrophotometric hydrazone method of Friedemann and Haugen (6). Blanks were run for each sample omitting the enzyme.

block the oxidation of malate which is present in great excess (*i.e.*, 100 μmoles). That this inactivity at pH 7.5 is actually referable to inhibition by traces of oxalacetate can be demonstrated by adding cyanide at the same pH. Under these conditions the oxidation of malate proceeds with a sevenfold increase in velocity (Table III).

Conversely, with the conjugated malic oxidase there is no significant inhibition by oxalacetate even when it is present in relatively large concentrations (Table IV). It will be noted that under the conditions of the test oxalacetate itself is not oxidized and therefore, the oxidation observed in the presence of oxalacetate is referable only to the malate. The argument might be raised that in the cyclophorase gel some mechanism exists for the destruction of oxalacetate so that no appreciable amounts can accumulate during the oxidation of malate. To test this possibility various amounts of oxalacetate (1–40 μmoles) wore incubated anaerobically with a cyclophorase gel for 5 minutes at 38°. Following deproteinization with trichloracetic acid, the oxalacetate was determined as total keto-acid by the hydrazone method of Friedemann and Haugen (5). Even with the smallest amount of oxalacetate there was an almost quantitative recovery as indicated in Table V. Estimation of oxalacetate as a keto-acid is valid since any pyruvate formed from oxalacetate is likewise inhibitory toward the dissociated malic oxidase (6). Thus, destruction or conversion of oxalacetate by the cyclophorase system cannot be the explanation for the lack of inhibition exhibited by the small amount of oxalacetate formed during the anaerobic oxidation experiment and certainly not when only a slight inhibition resulted from large quantities of oxalacetate being added to the system (Table IV). A confirmation of the view that the activity of the conjugated malic oxidase is unaffected by oxalacetate is provided by the fact that cyanide has no effect upon the reaction velocity.

At pH 9.5 the dissociated malic oxidase is much less inhibited by oxalacetate so that anaerobic Thunberg experiments can be carried out in the absence of a fixative. However, with increasing amounts of oxalacetate the oxidation of malate is arrested even at pH 9.5 (Table IV).

The data indicate that the inhibition by oxalacetate is not referable to equilibrium considerations. In the system represented above in equation 1 with 2,6-dichlorophenolindophenol as the dye, the calculated value of ΔF is—14 700 cal. (1), which, coupled with the initial high concentration of malate, would drive the reaction to completion. More likely, the oxalacetate may compete with the malate for position on the surface of the dissociated enzyme but not the conjugated enzyme.

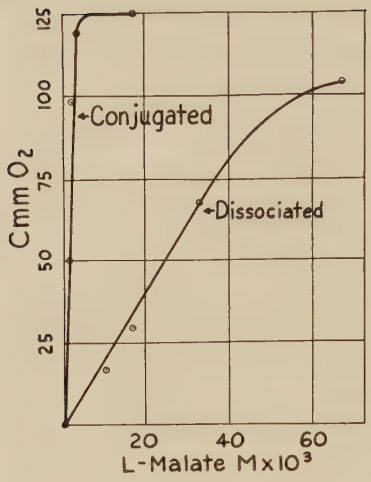

Fig. 3. For the conjugated experiment each manometer cup contained 1.0 cc of R_3K, 0.3 cc of 0.01 M adenylic acid, 0.2 cc of 0.02 M magnesium chloride, 0.1 cc of 0.1 M phosphate (pH 7.2), malate as specified in the figure, and water to make 3,0 cc. Oxygen in the gas space, alkali in the center wall.
For the dissociated experiment each cup contained 1.0 cc of R_3K, 350 μgm of DPN, 0.2 cc of 0.1 M arsenite, 0.5 cc of 0.1 per cent methylene blue, 0.5 cc of 0.1 M 2,2-dimethyl-1,3-propanediol buffer (pH 9.5), malate as specified in the figure and water to make 3.0 cc. Oxygen in the gas space, alkali in the center well.
Duration of the experiment, 10 minutes.

Effect of Substrate Concentration. The cyclophorase malic enzyme can oxidize vanishingly small amounts of substrate and over a fairly wide range of concentration the initial rate is nearly independent of substrate concentration as demonstrated in Fig. 3. At high concentrations the maximal rate declines due, presumably, to the malate itself acting like other salts in high concentration and effecting some dissociation.

Under similar conditions the dissociated oxidase, fortified with sufficient DPN to provide saturation shows a quite different substrate concentration *vs.* activity curve (Fig. 3). At extremely high concentrations of substrate the dissociated oxidase also becomes inhibited and the plateau of the curve bends downward.

The conjugated oxidase reaches maximum activity at 8×10^{-3} M and half-maximum activity at 2×10^{-3} M whereas for the dissociated counterpart the corresponding values are 65×10^{-3} M and 25×10^{-3} M, respectively.

Activity, for a given amount of substrate, has been measured by the oxygen uptake over the first 10 minute period. Longer periods would, of course,

vitiate the experiment since in the case of the low concentrations, the amount of available substrate would become exhausted during the test period.

The usual Thunberg procedure is not altogether suitable for demonstrating such a substrate concentration curve over a wide range, since too-rapid reduction times are not accurate owing to the variable time required to tip and mix the contents of the tube and too-slow reduction times are characterized by uncertain bleaching times. Accordingly, the test was carried out in aerobic test systems differing only in the final electron acceptor (with the conjugated the cytochrome -0_2 system was used; with the dissociated the cytochrome system was by-passed using methylene blue -0_2). It was necessary, in the experiment with the dissociated oxidase, to add methylene blue since treatment with arsenite or capryl alcohol not only dissociates the intact pyridinoprotein but also damages some other carrier in the electron transport chain between the substrate and oxygen.

DISCUSSION

In this system the reaction being studied is:

$$\text{malate} + \text{DPN}_{\text{ox.}} \rightarrow \text{oxalacetate} + \text{DPN}_{\text{red.}}$$

Green, *et al.* (7) have shown that oxalacetate is actually the product of oxidation but in the absence of cyanide a secondary reaction occurs:

$$\text{oxalacetate} \rightarrow \text{pyruvate} + \text{CO}_2$$

Recently Ochoa *et al.* (15) and Conn *et al.* (18) have described another malic enzyme found in pigeon liver and higher plants, respectively, which catalyzes the following reaction:

$$\text{malate} + \text{TPN}_{\text{ox.}} \rightarrow \text{pyruvate} + \text{CO}_2 + \text{TPN}_{\text{red.}}$$

Only the DPN-requiring system is found in kidney and liver cyclophorase. Whether the product is oxalacetate or pyruvate is immaterial since each cannot be further oxidized as such but must first undergo a condensative reaction. Thus, although the system is a *complex* of enzymes, the oxidative reaction studied here is essentially *one-step* since the experimental conditions preclude any major participation of the products in the observed oxidative activity.

The test system employed throughout was, with one exception, an anaerobic Thunberg system which couples the oxidation of malate with the reduction

TABLE VI

Comparison of the Properties of the Conjugated and Dissociated Malic Oxidase.

Property	State of Enzyme	
	Conjugated	Dissociated
Requirement for added pyridinenucleotide....	No DPN required	DPN required
Effect of 2,4-dinitrophenol, etc.	Inhibited	Not inhibited
pH Optimum	7.0–8.0	9.5
Effect of oxalacetate	Not inhibited	Inhibited
Substrate concentration, saturation	8×10^{-3} M.	140×10^{-3} M.

of a dye. Two enzymes mediate this electron transfer chain *viz.* the malic dehydrogenase, a pyridinoprotein, and diaphorase, a flavoprotein. Since the variable being tested is the pyridinoprotein it is obligatory that the diaphorase remain constant—a condition supported by our previous work (11) and other investigations (17).

In Table VI a comparison is made between the properties of the malic oxidase in the conjugated and dissociated states. It is interesting to note that the properties of the dissociated malic oxidase still attached to the particles almost exactly parallel those observed by other investigators (2, 6, 17, 19) using purified, soluble preparations.[1]

METHODS

The reagents and methods used in this investigation are identical with those described in previous papers (11, 12). Oxalacetate was estimated by the spectrophotometric method of Friedemann and Haugen (5).

ACKOWLEDGEMENT

This investigation was supported by a grant from the Commonwealth Foundation.

It is a pleasure to acknowledge the advice and encouragement of Professor David E. Green as well as the technical assistance of Irene Rechnitz.

SUMMARY

A comparison has been made between the properties of the conjugated malic oxidase as it exists in the cyclophorase system and of the same oxidase after it has been converted to the dissociated state.

[1] If the data in Fig. 1 are replotted according to the Lineweaver-Burk double reciprocal method (J. Am. Chem. Soc., *56*, 658 (1934)), K_m for the dissociated malic oxidase (on the particles) is found to be 2.5×10^{-5} M. This is in good agreement with the results of Feigelson, P., Williams, J. N. Jr., and Elvehjem, C. A. (J. Biol. Chem., in press) who have determined K_m to be 3.7×10^{-5} M. for DPN and the purified, soluble malic dehydrogenase.

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ELECTRON MICROSCOPY OF MYELIN SHEATH IN SECTIONS OF SPINAL CORD[1]

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The well-known limitations of the light microscope in terms of resolving power have always precluded the possibility of verifying by direct observation any concepts concerning lipid-protein organization in the myelin sheath derived from polarization or X-ray diffraction data. With the greatly increased resolution offered by the electron microscope, it is natural that intensive efforts would be made to subject nerve tissue to electron-optical analysis against the background of current biophysical thought. The relatively poor penetrating power of the electron beam has, however, imposed such strict limitations on specimen thickness that for some time the electron microscopist was restricted to the study of submicroscopic tissue fragments dissociated in various ways. Although dissociation methods have proved of great value in the study of some reasonably homogeneous materials such as collagen, the complex ultrastructure of the nervous system can be most adequately studied by the use of sections, in which the spatial relationships of diverse tissue elements are preserved.

Only recently have the technical advances made in sectioning permitted even a start on the analysis of important problems relating to submicroscopic organization in nerve. Methods of specimen preparation for electron microscopy, particularly as regards fixation, have been largely borrowed from the empirical techniques of histology, and are still far from satisfactory. Nevertheless, it is possible to secure sections that are thin enough to reveal detail far below the resolution limit of the light microscope. This means that progress toward solving the problem of fixation can go hand in hand with advance in knowledge of nerve ultrastructure as various artefacts are encountered and either eliminated or viewed in proper perspective.

[1] This study was aided by a grant from the Graduate Medical and Cancer Research Fund of the University of Minnesota.

A beginning has been made in the electron microscopy of peripheral nerves in section (1, 8, 10). The present communication deals with the structure of the myelin sheath in the central nervous system as revealed in sections of the spinal cord.

MATERIAL AND METHODS

The material consists of small pieces of lumbar cord from normal adult male rats. Fixation was made by perfusion with neutral 10 per cent formalin, followed by washing for 12–15 hours in running water, then by immersion of tissue blocks in 2.0 per cent osmium tetroxide. The samples of tissue used were small, measuring no more than 4 mm on a side. After fixation, the tissues were dehydrated through a graded series of alcohols and embedded in n-butyl methacrylate according to the method of Newman, Borysko and Swerdlow (4, 5), using 1.5 per cent benzoyl peroxide to catalyze the polymerization. Sections were cut on a Spencer No. 820 microtome adapted to feed in increments of 0.1 micron. The adaptation of the microtome followed the suggestion of Pease and Baker (6) and was performed in the University of Minnesota shops. A glass cutting edge (3) and a water trough to float the sections (2) were used throughout. The modified microtome feed gave sections that appeared fairly uniform in thickness in the electron microscope, and were useful for observing many details. Since, however, the degree of resolution is highest in the thinnest sections, two additional expedients were employed for obtaining extremely thin sections or thinner areas of individual sections. The first consisted in cutting a series of 10–15 sections with the microtome set to feed at 0.1 micron. The feed mechanism was then disengaged, after which 3 or 4 sections, noticeably thinner than the rest, could be cut. This effect is presumably due to slight longitudinal compression of the methacrylate during the cutting cycle with the feed mechanism functioning, followed by a slow expansion of the block to give a few thinner sections after disengagement of the feed. This method is in principle the same as the thermal expansion technique of Newman, Borysko and Swerdlow (4). The second expedient follows the reasoning of Richards, Anderson and Hance (7) and involved altering the position of the tissue block in the microtome after it had been faced with the knife, so that wedge-shaped sections were cut. There was a noticeable increase in resolution in working from the thick to the thin end of such sections.

It will be appreciated that the actual thickness of the sections is of academic interest only, and that in the last analysis the quality of the final image is the sole criterion of suitable section thickness, other things being equal. Furthermore, the allowable thickness for good resolution may vary with different types of material, different embedding media, etc. Hence no attempt was made to secure absolute values for section thickness in this work, and the only statement that can be made in this connection is that the sections yielding the best resolution were in all probability well below 0.1 micron in thickness.

The embedding medium was allowed to remain in the sections, because earlier attempts by the writer to dissolve out the methacrylate with various solvents at temperatures ranging from room temperature to —40° were attended with the

appearance of large-scale distortion. This disadvantage more than overshadowed the gain in contrast and resolution following removal of the plastic, and reliance was placed on the above-mentioned expedients for obtaining the thinnest possible sections.

Sections were floated on distilled water in the knife trough as they were cut, and then removed in groups to dishes of distilled water by means of a camel's hair brush. Formvar-coated nickel grids were used as specimen supports. A grid, held in fine forceps, was immersed vertically in the distilled water and a section was drawn or pushed with a fine needle until one edge was in contact with the grid.The latter was then carefully withdrawn in a vertical direction, with the result that the section flattened evenly against the formvar membrane. The sections were allowed to dry in air and were then studied with the RCA electron microscope, model EMU, having a biased gun. No objective aperture was used, but a 13-mil aperture[1] was in place in the anode throughout most of the work.

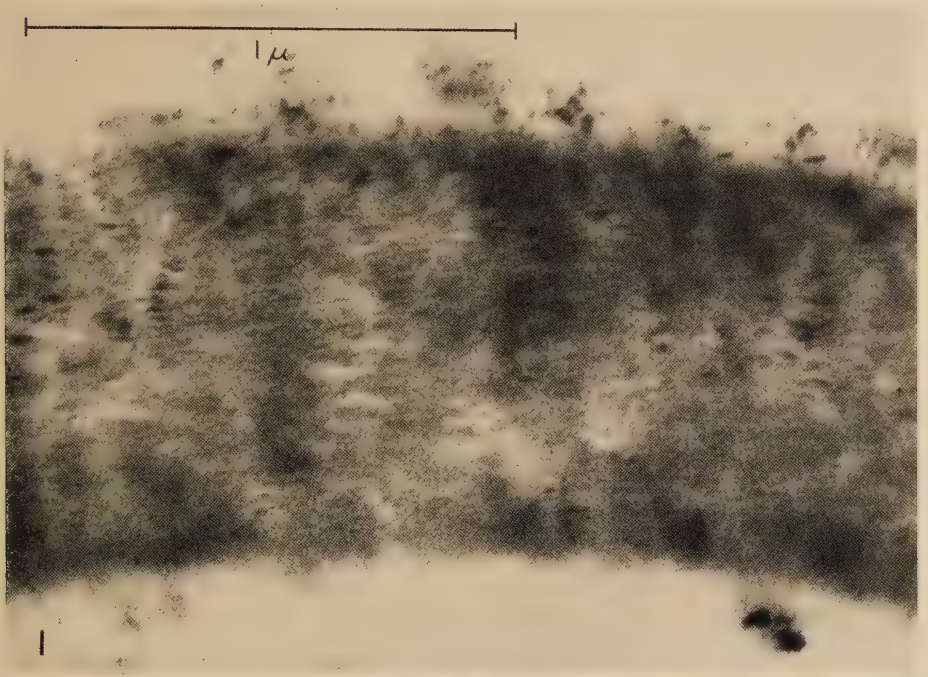
OBSERVATIONS AND DISCUSSION

The most striking feature of the myelin sheath as observed in this study is a concentric arrangement of thin plates or placodes of varying electron density (Fig. 1). The denser plates measure approximately 120 A in thickness, while the less dense ones average 61 A thick. The width of both kinds of plates as measured in cross sections ranged from 540 A to 1150 A, and the length as seen in longitudinal sections was found to lie between 900 and 1700 A. There was no correlation between the thickness of the plates and their length or width. These values for width and length are extremely small in the light of a previous interpretation of the myelin sheath in peripheral nerve (1) that assumes the sheath to be made up of thin concentric cylinders of indeterminate length. It is imperative, therefore, that electron-optical effects be considered in interpreting the appearance of Fig. 1 and 2. The appearance of placodes in these figures is in all probability not due to thermal drift of the specimen; first, because images of isolated granules in the field are not drawn out into streaks. Secondly, thermal drift is ordinarily unidirectional and thus could not account for the concentric arrangement of the plaques as seen in Fig. 1. Finally, the amount of thermal drift that would

Fig. 1. Portion of cross section of a large nerve fiber in the spinal cord of the rat, showing placodes concentrically arranged in the myelin sheath. Magnification: 64 500 ×.

Fig. 2. Longitudinal section of a fine nerve fiber in the spinal cord of the rat. Part of the axis cylinder appears at the bottom of the figure, and above it is the darker myelin sheath, separated into two layers by an oval space. The space is interpreted as an artefact of embedding, but the disruption has separated individual plaques somewhat so that they are more easily visible. Magnification: 50 300 ×.

[1] Designed by James F. Marvin of the Department of Biophysics, University of Minnesota.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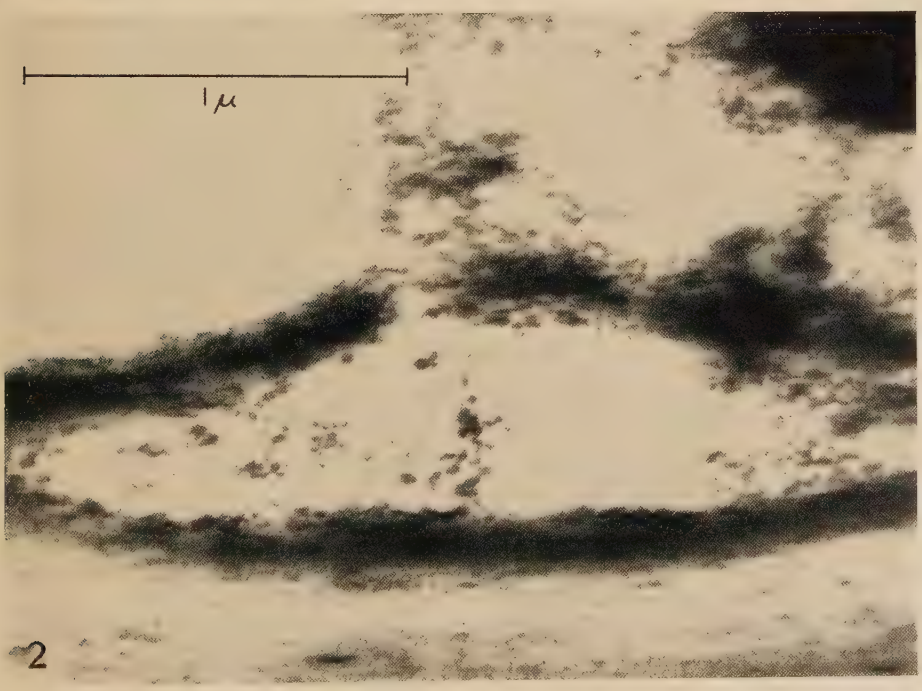
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produce the appearance in these figures would almost certainly be all but imperceptible on the final viewing screen, whereas a concentric streaking was clearly and frequently observed in the thinnest cross-sections before the photographic record was made. As a routine precaution, beam intensity was always kept at the lowest possible value consistent with recognition of structures or areas on the final viewing screen.

The difference in thickness, as well as in electron density, between the two types of plaques may possibly be due to uneven penetration or adsorption of osmium tetroxide, rather than to inherent differences in composition of the plaques. The fact that there is no discernable regularity in the relative distribution of the two types makes one further disinclined to regard them as discrete entities. Nevertheless, it is interesting to note that the total of the two averages, 181 A, is close to the value of 180 A for the fundamental spacing of lipid-protein layers deduced from the X-ray diffraction data of Schmitt, Bear and Palmer (9). Fernández-Morán (1) describes unit lamellae in sections and in dissociated preparations of peripheral nerves. Evidence from some of his sections suggests that the irregularly polygonal plates in the fragmented material represent portions of previously intact, concentric myelin cylinders. The results of the present study do not bear out this concept, since no sections were seen in which there was complete continuity of lamellae around the fiber axis. It is of course necessary to use extreme caution in interpreting ultrastructure in sectioned material that is in all probability poorly fixed from the standpoint of electron microscopy. Accordingly, the possibility is readily admitted that fragmentation of myelin cylinders into plates may have occurred as an artefact of preparation in the present work. If this is the case, it seems likely that the fragmentation would have occurred during fixation rather than in embedding, because the individual placodes preserve their flat shape despite a type of distortion that sometimes occurs in the polymerization of the plastic embedding medium (Fig. 2). When the degree of such distortion is not excessive, it is sometimes an advantage, inasmuch as it produces enough separation of tissue components to aid in the interpretation of intact structures.

A second explanation for the appearance of plaques rather than of continuous concentric lamellae suggests itself; namely, the possibility that the myelin sheath may show a different organization in the central nervous system than the one to be found in peripheral nerve. However, Fernández-Morán's evidence for the existence of continuous myelin cylinders is not entirely conclusive, and further work will obviously be necessary to clear up this point. It should be mentioned that the plates found in the present

work show a tendency toward imbrication in both the concentric and longitudinal directions. This agrees with Fernández-Morán's finding of a scaly surface texture in replica-adhesion preparations of the sheath. It therefore seems likely that the plates of this study correspond to the scales appearing in replica-adhesion preparations, despite the fact that only the thicker type fall within the 100–200 A range given by Fernández-Morán for the thickness of the scaly structures imaged in his replicas. The tendency toward overlapping observed here is such as to mask any interposition of complete concentric cylinders between the plaques of the sheath. Still, an average spacing of 90 A between individual plaques in the radial direction found by the writer (by direct measurement of cross-sections) is in fair agreement with Fernández-Morán's predominant value of 80 A for thickness of structures designated by him as unit lamellae, as determined by measurement of shadowed preparations.

The great majority of sections seen in this study show the myelin sheath to be a fairly compact structure, displaying neither the moderate-sized oval spaces between myelin rings described by Fernández-Morán (1), nor the large irregular spaces appearing in the sheath in the micrographs of Rozsa et al. (8). Sections from a few blocks of tissue were encountered in which the myelin sheaths were distorted in shape and exhibited irregular spaces ranging from small gaps in the myelin to gross internal disruption of the entire sheath with radial compression of the axis cylinder. These appearances were the exception rather than the rule, however, and are interpreted as an aberrant phenomenon in the embedding process. Newman, Borysko and Swerdlow (5) have commented on the fact that some tissue blocks become swollen and distorted during embedding. Despite the writer's attempts to standardize the embedding procedure throughout this study, a few blocks in every batch of tissue showed bubbles in the plastic and easily discernable deformation of the tissue. Such blocks were of course discarded, but it is considered likely that the spaces seen occasionally within the myelin sheath in this work are an expression of small-scale distortions essentially similar in nature to those macroscopically visible in poorly embedded blocks.

SUMMARY

1. The structure of the myelin sheath in the central nervous system has been studied in sections of the spinal cord of the rat.

2. The sheath has been found to be composed of concentrically arranged placodes of varying electron density, denser types averaging 120 A in thick-

ness and less dense types averaging 61 A. The width of both kinds of plaques was found to vary unsystematically between 540 A and 1150 A, and their length was found to range between 900 A and 1700 A.

3. The placodes in the myelin sheath are imbricated in both the concentric and longitudinal directions so as to mask any possible interposition of complete concentric cylinders between them.

4. Open spaces in the myelin sheath comparable to those described by other authors for peripheral nerve were seen only occasionally, and are here interpreted as artefacts of embedding rather than of fixation.

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BRIEF NOTES

DOES ADENOSINETRIPHOSPHATE CAUSE A CONTRACTION OR A DISAGGREGATION OF DISSOLVED ACTOMYOSIN?[1]

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Received June 17, 1950

Through the investigations of A. Szent-Györgyi (13, 15, 9), it has been recognized that muscular contraction is due to the effect of interactions between myosin, actin, ATP and inorganic cations. Indeed, contraction phenomena are displayed, upon addition of ATP, by various actomyosin systems such as washed muscle fibers, actomyosin threads, or suspended actomyosin flocks (13, 16, 8).

Addition of ATP to a solution of actomyosin causes a drop in viscosity (2, 1, 5, 7). Without quantitative information, this could be regarded as indicating either a disaggregation or a contraction of the dissolved actomyosin. The Szent-Györgyi school has always assumed disaggregation, without contraction. The main arguments for this are *1.* observations on changes in light-scattering (5, 6) and *2.* the imitation of the ATP effect by urea and other disaggregating substances (6).

However, the author's early investigations on light scattering cannot be regarded as final since no means were available to exclude the presence of submicroscopic particulate matter, whereas also the theory at that time (5) was not sufficiently developed to permit quantitative conclusions. An extensive investigation concerning the turbidity of myosin systems is now in progress. It must be remarked that just the latter type of measurement has induced Jordan and Oster (3) to regard the effect of ATP as a true contraction of dissolved actomyosin. However, the author's optical investigations, to be published elsewhere, do not confirm this view.

The present communication presents two other arguments, resulting from viscosimetric and ultracentrifugal investigations.

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[1] Read at the meeting of the Southeastern Section of the Society for Experimental Biology and Medicine, January, 1950.

[2] With support of the Rockefeller Foundation.

[3] With support of the American Heart Association and of the National Heart Institute.

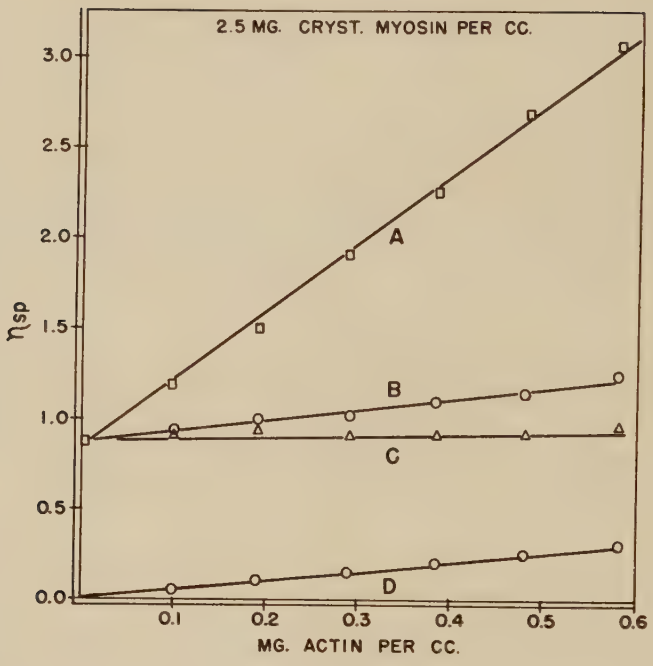

Fig. 1. Viscosities of actin-myosin systems in neutral 0.5 molar KCl. Explanation, see text.

EXPERIMENTAL METHODS

Myosin was initially prepared by crystallization, following the method of Szent-Györgyi (14). The ultracentrifugal experiments, however, were done with the monodispersed crystalline product, prepared according to Mommaerts and Parrish (10).

Actin was prepared according to the original method of Straub (12), employing an additional extraction of the acetone powder with ether.

ATP was prepared in the Beirut laboratory with the method of Kerr (4); later, a commercial preparation of the Ba-salt was used. All solutions were purified by filtration through a cation-exchange column (11).

Viscosity determinations were performed as in previous studies (7). Ultracentrifugal sedimentation was measured in the analytical ultracentrifuge model E, built by the Specialized Instruments Corporation, Belmont, California.

VISCOSIMETRIC MEASUREMENTS[1]

The results of an experimental series are given in Fig. 1, in which the specific viscosities of several systems are indicated. The basic constituent in all experiments

[1] (Beirut, 1947.)

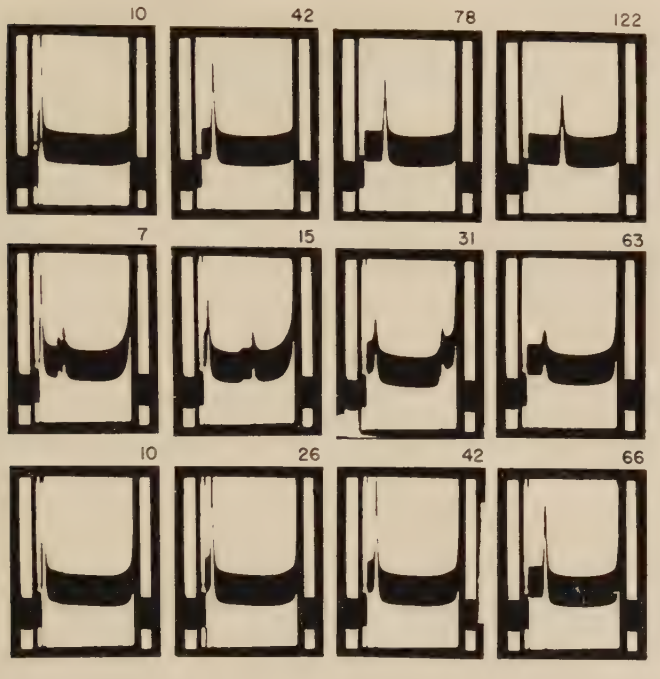

Fig. 2. Ultracentrifugal sedimentation patterns of actin-myosin systems in neutral 0.5 molar KCl, containing 0.01 molar $MgCl_2$. Myosin concentration 1 mg per cc. throughout. Upper row: pure myosin. Second row: actin added (about 0.2 mg in terms of total protein). Third row: idem, in presence of ATP. Time of sedimentation at 42,000 r.p.m. indicated in minutes, on the top of each diagram.

is crystalline myosin, 2.5 mg per ml of 0.5 molar neutral KCl. The specific viscosity of this solution was 0.86. Experimental solutions contained various amounts of actin. The amounts added are indicated on the abscissa, in terms of the total protein of the actin solution. The degree of purity of the actin is not known.

Curve A gives the viscosities of the resulting actomyosins, whereas curve B gives the values for the same solutions after the addition of an excess of ATP in 0.5 molar KCl and correction for the slight dilution caused by this addition.

Curve D indicates the viscosities of solutions containing the same amounts of polymerized actin, but no myosin, measured under the same conditions. When the points of this experiment are substracted from those of graph B, curve C is obtained. Except for a very slight slope, to be ascribed to the concentration effect of the viscosity, this is essentially a horizontal straight line, corresponding to the viscosity of the myosin alone.

This experiment shows therefore that, in the presence of ATP, the viscosity of an actomyosin is exactly the sum of the viscosities of the individual components, myosin and actin.

ULTRACENTRIFUGAL OBSERVATIONS[1]

Fig. 2 illustrates the results of a relevant ultracentrifugal experiment. The upper row of Philpot-Svensson diagrams shows the sedimentation of myosin. The sedimentation constant at this concentration approximates $S_{20,w} = 6.0$. In the second row, the sedimentation patterns are shown of the same amount of myosin, to which an amount of actin is added, which is insufficient to engage all the myosin in actomyosin formation. The actomyosin which, for reasons as yet unexplained, consists of two components, sediments very rapidly. It leaves a reduced amount of free myosin sedimenting at its original speed.

Upon addition of ATP (third row), the actomyosin disappears completely, and the original free myosin is entirely restored. In both experiments with added actin, a polydispersed slowly moving boundary is present, due to impurities in the actin. The actin itself is not revealed in the third row of patterns, since it is present in a very small amount and reaches the bottom of the cell during acceleration.

DISCUSSION AND SUMMARY

The two types of experiments recorded offer independent evidence for the disaggregation of dissolved actomyosin by ATP. The viscosimetric study shows that in the presence of ATP, the flow characteristics of actomyosin are the same as can be computed by addition of the viscosities of the myosin and actin separately. Ultracentrifugally, a liberation of the free myosin from actomyosin by ATP is clearly demonstrated.

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THE CONDUCTION TIME OF THE BLOCK TO POLYSPERMY IN THE SEA-URCHIN EGG

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After insemination of the sea-urchin egg (*Psammechinus miliaris*), a change in cortical structure is propagated over the egg surface, the conduction time being about 20 seconds at 18° C. This is recorded most easily under dark ground illumination though it can also be seen with polarized and normal light (1). The time relationships of this cortical change are not inconsistent with the hypothesis that it is caused by the diffusion of a substance, m. w. app. 20,000, directly or indirectly derived from the sperm head, through the egg cytoplasm (2). If this cortical change is the block to polyspermy, it follows that the probability of a successful sperm-egg collision must be low, for at normal sperm densities, e.g. 10^6 or 10^7 sperm/ml., an egg will sustain 10–100 potentially successful sperm-egg collisions during the propagation of the cortical change (1). The following experiment, carried out at 18° C, strongly supports the view that the block to polyspermy is established slowly, i.e. in a matter of seconds rather than a fraction of a second, and that the probability of a successful sperm-egg collision is low. Spermatozoa were added to a vessel containing unfertilized sea-urchin eggs so that the final sperm concentration was 3×10^6/ml. After the eggs had been in contact with this sperm suspension for 25 seconds, the spermatozoa were suddenly killed with hypotonic sea water, but the eggs were transferred to normal sea water and allowed to develop. Counts at first cleavage gave the following results: monospermic eggs, 85 per cent; unfertilized eggs, 13 per cent; polyspermic eggs, 2 per per cent. In a second vessel exactly the same procedure was carried out, except that instead of killing the spermatozoa after they had been in contact with the eggs for 25 seconds, more spermatozoa were added bringing the final sperm concentration up to 3×10^8/ml. Counts at first cleavage in this experiment gave the following results: monospermic eggs, 54 per cent; unfertilized eggs, 2 per cent; polyspermic eggs, 44 per cent. This shows that nearly half of the eggs which were fertilized in 25 seconds had not finished propagating their block to polyspermy in that time and became polyspermic because of the new sperm bombardment they received after the 25 second period was terminated.

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THE SPECIFICITY OF RIBONUCLEASE AND OF DEOXYRIBONUCLEASE

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Recent studies (3, 7, 8) of the degradation of deoxyribonucleic acid with deoxyribonuclease have shown that the action of this enzyme closely parallels the action of ribonuclease on ribonucleic acid (1, 10, 11). In each case there occurs an initial depolymerization of the complex nucleic acid molecule into smaller units which are then hydrolysed, by cleavage of internucleotide phosphate linkages, to acid-soluble polynucleotides of varying molecular size. Much evidence exists which indicates that both ribonuclease (5, 10, 11) and deoxyribonuclease (8, 13) preferentially hydrolyse the phosphate linkages of pyrimidine nucleotide groupings of the respective substrates and leave a relatively large, non-diffusible "core" which contains an increased purine-pyrimidine ratio and which is resistant to further enzyme degradation.

This distinct analogy between the modes of action of ribonuclease and of deoxyribonuclease becomes of increased interest when considered in relation to the specific action of the enzymes. Thus, as is well known, deoxyribonuclease has no action on ribonucleic acid, and ribonuclease is without effect upon deoxyribonucleic acid. In consequence of the facts outlined above, it appeared of some interest to determine whether, on the assumption that both ribonucleic acid and deoxyribonucleic acid contain some similar inter-nucleotide phosphate linkages, the specificity of the enzymes was conditioned by the degree of polymerization of the substrates.

The ribonuclease used in the work summarized below was isolated from beef-pancreas according to the method of Kunitz (4). Although the preparation contained no demonstrable alkaline phosphate or deoxyribonuclease activity, solution of the enzyme were invariably adjusted to pH 4.0 and heated at 100° for 3–5 minutes, in order to obviate the possibility that minute traces of deoxyribonuclease accompanied the ribonuclease.

Deoxyribonuclease also was isolated from beef-pancreas according to the method of McCarty (6), additional purification being obtained by repeated fractional precipitation as described by Overend and Webb (8). The enzyme preparation was free from ribonuclease and phosphatase activities, but exhibited a weak proteolytic action against a casein substrate.

In preliminary studies, designed to determine whether ribonuclease was able to hydrolyse the partially degraded products resulting from the controlled action of deoxyribonuclease on deoxyribonucleic acid, attempts were made to inhibit the action

of the deoxyribonuclease at convenient stages during the degradation by the addition of suitable specific inhibitors (2), and then to carry the hydrolysis further by the addition of ribonuclease. The variable results obtained in these experiments, however, suggested that the inhibitors used had little or no effect upon the activity of deoxyribonuclease already in combination with its substrate. Accordingly, the effect of the addition of ribonuclease to a solution of deoxyribonucleic acid containing a sub-optimal concentration of deoxyribonuclease was investigated. Control experiments showed that the addition of further deoxyribonuclease to such a system resulted in a marked increase in the rate of hydrolysis. The experimental procedure was as follows:— A mixture of 0.1 per cent thymus deoxyribonucleic acid (20 ml), 0.01 M magnesium sulphate (10 ml) and 0.1 M veronal buffer, pH 7.0 (10 ml) was brought to 37° and a solution of deoxyribonuclease (0.001 per cent, 10 ml), preheated to 37°, added. The extent of the hydrolysis, in terms of the increase in acid-soluble "organic" phosphorus, was measured at suitable time intervals in aliquots (5 ml) of the solution according to the method previously described (8). After 20 minutes, the enzyme-substrate mixture was divided into two fractions, to one of which was added a solution of ribonuclease (1 per cent) to give a final concentration of 0.1 per cent. From each of these solutions, incubated at 37°, aliquots (5 ml) were withdrawn at suitable time intervals and analysed for acid-soluble organic phosphorus. Such experiments, and similar determinations in which ribonuclease was added to a fraction of the deoxyribonuclease—deoxyribonucleic acid mixture after 10, 30, 45, 50 and 60 minutes revealed that in each case the presence of ribonuclease failed to influence the rate of the hydrolysis of the deoxyribonucleic acid.

In subsequent experiments, mixtures identical with those described above were incubated at 37° for 10, 20 and 45 minutes, cooled to 0° and the enzyme protein then removed by the Sevag (12) method. This procedure invariably resulted in the loss of nucleic acid from the solution, presumably as the enzyme-substrate complex, since little loss of nucleic acid occurred when either heat-inactivated deoxyribonuclease, or ribonuclease, was added to solutions of deoxyribonucleic acid and then removed by the Sevag method. The mixed deoxyribo-polynucleotides remaining in solution after the above treatment, while readily degraded further by the addition of deoxyribonuclease, were completely resistant to the action of ribonuclease. Similarly, when yeast ribonucleic acid was partially degraded by a short treatment with ribonuclease, and the enzyme protein then removed as before, the resulting products were readily digested with ribonuclease, but were resistant to the action of deoxyribonuclease. The fact that the products obtained after deproteinisation, in each case, were hydrolysable by the appropriate enzyme, proved that the inability of ribonuclease to hydrolyse the partially degraded deoxyribonucleic acid, or of deoxyribonuclease to hydrolyse the partially degraded ribonucleic acid, was not due to any inhibitory action of residual traces of chloroform or octyl alcohol in the aqueous solutions.

Finally, a series of fractions consisting of mixed polydeoxyribonucleotides of increasing mean molecular weight, and isolated by fractionation of the products resulting from the action of deoxyribonuclease on deoxyribonucleic acid (8), when treated with ribonuclease (0.1 per cent) at pH 7.0 and 37°, proved completely resistant to the action of the enzyme.

These results show that ribonuclease and deoxyribonuclease are completely specific

for ribonucleic acid and deoxyribonucleic acid respectively, and that the specificity is not conditioned by the degree of polymerization of the substrates.

A tentative hypothesis to account for these facts has been published elsewhere (9).

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CELL PERMEABILITY TO ELECTROLYTES IN TISSUE CULTURE

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In comparing the action of various electrolytes upon cells in tissue culture the ascending lyotropic series: SO<Br<Cl<I<SCN<OH can easily be established. In the following experiments the lyotropic anion series can be demonstrated. On the second or third day of cultivation in a liquid medium, the medium is removed and replaced by a new medium consisting of a serum-isotonic solution of NA_2SO_4, NaBr, NaCl, NaI, NaSCN or NaOH. Neutral red solution 1/20,000 may be added in order to distinguish more details. In the NA_2SO_4 medium conspicuous signs of cell shrinking will appear, much less so in cultures of the NaBr or NaCl medium. In the medium of NaI (or KI) considerable cell swelling occurs: in the cytoplasm vacuoles appear, the cells successively accept the form of spherules, and lose the vital neutral red granula. Cell swelling of the same kind but in much higher degree occurs in the NaSCN (or KSCN) medium. Most conspicuous cell swelling can be found in a NaOH medium beginning at pH 11.

In the following experiments the cover glass method, and cultures from 8–9 days old chicken embryos were used. The medium consisted of equal parts of concentrated embryonic juice and amniotic fluid of the same embryos. This culture medium gives excellent growth, and can be any time for experimental purposes completely removed and replaced by other media.

On the third day of growth of fibroblasts preferably from embryonic heart tissue, the normal culture medium was removed and the cultures were put in a medium consisting of n/7 solution of NaI, KI, NaSCN or KSCN with addition of 1/20,000 solution of neutral red. An equal number of other cultures of possibly equal growth conditions, having been brought likewise in a new medium of n/7 solution of NaI, KI, NaSCN or KSCN and 1/20,000 neutral red, was put at the same time in anaerobic conditions. This was attained either with hydrocyanic acid, or stopping of oxidation was also achieved by filling up the hollow of the slide with paraffin liquid at room temperature and carefully avoiding air bubbles while closing the hollow with the cover glass.

It has been found as a rule that cultures kept in aerobic conditions show the above described cell swelling considerably earlier—with an average difference of 50 minutes—(Fig. 1)—than cultures living in anaerobic conditions (for which only glycolysis provides growth energy), (Fig. 2). At a time when most of the cells of cultures in aerobic conditions are considerably changed by swelling, with signs of beginning cytolysis (i.e. loss of vital granules, spherical cell forms, visible Brownian movement of large cytoplasmatic particles),—cultures living in anaerobic conditions are still unchanged and show normal vital staining. It may thus be concluded, that the

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This work was begun before the war at the Tissue Culture Laboratory, Physiological Department (Head, Prof. H. Grossfeld), Lwów University.

anions I, SCN permeate into tissue cells kept under aerobic conditions more rapidly than into cells under anaerobic conditions, the conspicuous sign of their permeation being the above described cell swelling.

DISCUSSION

It is not easy to define whether this cell swelling is caused by the hydration of cell particles under the influence of the hydrating action of the anions I, SCN in accordance with their position in the lyotropic series of anions, or the cell swelling is a result merely of the rapid osmotic penetration of those anions into the cell: in the first case we are confronted with molecular attraction forces, and in the second case with diffusion phenomena (molecular motion). For our present purpose, however, this distinction may be temporarily disregarded.

Now the results show that cell respiration promotes the penetration of anions from the medium into the tissue cell, and that evidently, cell permeability to anorganic salts is connected with metabolic processes of the cell. However it is yet premature to determine whether cell respiration is only accompanied by penetration of anions, or cell respiration is also stimulated by the accumulation of anions.

The correlation between respiratory metabolism and the penetration of anions into the cell may be assumed as follows: Penetration of anions into the cell may be promoted by the active iron of the system cytochrome-cytochromoxidase. The change of valency of the iron atom in the hemin molecules must involve the motion of anions, because the trivalent iron atom attaches one anion more than the bivalent iron atom does. Or in other words, the motion of anions is connected with continuous transport of electrons from one atom to another in consequence of the wave-like change of valency of the iron atom in the cytochrom systeme. The anions thus move from points of higher oxidation power (ox.-side, Fe^{+++}) to points of lower oxidation power (red.-side, Fe^{++}). The cell should then show in general the tendency to transfer anions from the cell surface to the interior of the cell (3). This cell activity should be more conspicuous in growing cells, and for this reason embryonic cells in tissue culture were chosen for these experiments. The results thus show that the fact of the respiratory accumulation of anions, of "anion respiration" (3 a. o.) may be referred also to animal tissue cells. It may also further be concluded that, if cell respiration causes motion of anions, the formation of potential differences of ionic origin could thus be involved, because the conditions of motion in the cell are different for cations and for anions.

SUMMARY

1. A method for demonstrating the lyotropic anion series on living tissue cells is described.

2. Cell respiration accelerates the motion of anions into the cell. Respiratory accumulation of anions seems thus to be a general phenomenon concerning also animal tissue cells.

3. Cell permeability to electrolytes is connected with cell respiration and may be regarded as part of metabolic cell activities.

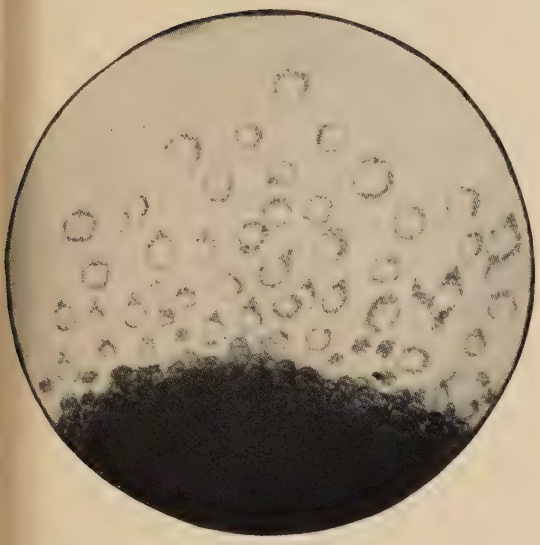

Fig. 1. Culture of chicken embryo heart fibroblasts brought into a n/7 solution of NaI in aerobic conditions, after 40 minutes. Beginning cytolysis.
Fig. 2. Culture of chicken embryo heart fibroblasts brought into a n/7 solution of NAI in anaerobic conditions after 40 minutes. Cells not altered.

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DEMONSTRATION OF A JELLY-SPLITTING ENZYME AT THE SURFACE OF THE SEA-URCHIN SPERMATOZOON

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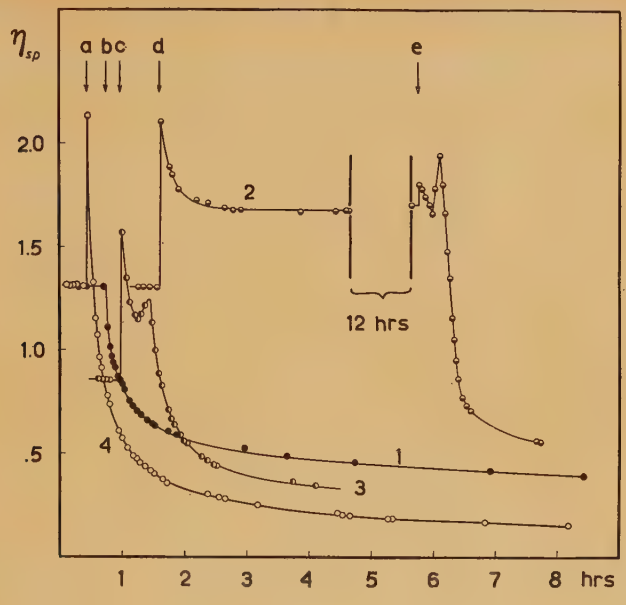
In order to reach the surface of the egg and to fertilize the egg, the sea-urchin spermatozoon has to force its way through the jelly coat surrounding the egg. It may be that this is accomplished, not only by the boring movement of the spermatozoon and the dissolving power of the carbon dioxide given off by the sperm (5, 6, 7) but also by the action of an enzyme at the surface of the spermatozoon. There are several indications that the sperm is able to break down enzymatically the jelly coat and fertilizin, the agglutinating principle of egg-water (i.e. sea-water in which ripe sea-urchin eggs have been kept for some time). A solution of the jelly coat agglutinates sperm of the same species reversibly, increases their motility, oxygen uptake (6) and also carbon dioxide output (7) and maintains their fertilizing capacity for a long period of time (9). Egg-water shows similar properties.

Monroy and Ruffo (2, 3) and Lundblad and Monroy (1) have found extracts of sea-urchin sperm to dissolve the jelly coat and to split the jelly substance and hyaluronic acid. The results of chemical analyses of the jelly substance (4, 8) disclose, however, that these effects cannot be attributed to the same principle, since the jelly substance contains neither hexuronic acid nor hexosamine, the building units of hyaluronic acid. Recent studies with sperm extract of *Paracentrotus lividus* made according to Lundblad and Monroy (1) indicate that the viscosity of jelly solution (for preparation, see 4) is lowered by sperm extract (added at b, curve 1) on account of a combination of the nuclear material of the sperm with the jelly substance, whereby the viscosity-giving elements are dehydrated and condensed, a fibrous precipitate being formed. It seems doubtful, whether any enzymatic splitting of the jelly substance occurs in this case.

When live *P. lividus* sperm are added (at d, curve 2) to a jelly solution of the same species, they are agglutinated strongly, giving rise to an apparently very high viscosity. After one or two minutes, especially if air is bubbled through the viscosimeter to quicken the dissolution of the clusters, the viscosity begins to drop gradually until a value is reached corresponding to the viscosity of the jelly solution + the sperm suspension without interaction. After this the viscosity may remain unaltered for many hours. If, however, a little octylalcohol is added (at e, curve 2) (one drop to 3 ml jelly solution is sufficient) some minutes or even several hours after the addition of the sperm, the viscosity increases again and then decreases slowly for some



minutes. At this moment a breakdown of the jelly substance, usually preceded by a new increase in the viscosity, sets in like a trigger reaction.

If octylalcohol is added (at c, curve 3) just before the addition of the sperm (also at c, curve 3), the effects of sperm agglutination and octylalcohol action are superimposed. Benzene, chloroform, and pyridine are without any effect. Heptylalcohol acts similarly to octylalcohol but more weakly. To begin with, the effect of amylalcohol is akin to that of the higher alcohols, since after the first peak due to the reversible agglutination a new increase is obtained, but the viscosity drops very slowly afterwards and no breakdown of the jelly substance is obtained. If, however, chloroform is added after a period of amylalcohol action, the jelly substance is broken down immediately.

The addition of living sperm (at a, curve 4) to a jelly solution to which octylalcohol has been added an hour or more before[1] first brings about the usual strong increase in viscosity owing to agglutination, but then the breakdown of the jelly substance follows instantaneously. The course and the rapidity of this liquefaction demonstrate that an enzyme responsible for this action must be situated at the surface of the sperm. Furthermore, these experiments indicate that the octylalcohol acts on the jelly substance and not on the enzyme, probably inactivating groups which inhibit the enzymatic action, the efficiency of the alcohol being due to the alcohol group and the length of the carbon chain. The amyl group is not big enough to block the inhibitory group, but is enlarged by the chloroform molecules due to association.

[1] Octylalcohol alone does not alter the viscosity of the jelly solution.

The experiments were carried out at about 18° C, which is well within the physiological range for *P. lividus*.

The explanation of these phenomena may be as follows. When spermatozoa are added to a jelly solution, they combine with the jelly substance in such a way that two or more spermatozoa react with the same high-molecular jelly complex, each spermatozoon reacting with several complexes. Thus the agglutination clusters are formed. The jelly-splitting enzyme, situated at the surface of the sperm, takes part in this reaction, forming an enzyme-substrate compound. By the destruction of the substrate the agglutination is reversed. But the fragment of the jelly substance which was in contact with the enzyme and by its action split off from the remainder of the jelly substance will not be detached completely from the enzyme, since some groups of the split-product are fixed to other parts of the surface. Thus the enzymatic action will be blocked by these shielding split-products. If, however, octylalcohol is added at this stage, the alcohol will filter in between the surface of the sperm and the shielding fraction of the jelly, combining with the inhibitory groups, thus inactivating them and loosening the split-product from the surface of the sperm. The enzyme is now free and can combine with a new portion of the high-molecular jelly substance—indicated by an increase in viscosity—and since the inhibitory groups also of this part have been inactivated by the alcohol, the breakdown of the jelly substance can proceed rapidly.

Now the question arises, what substance acts instead of octylalcohol in Nature. Perhaps the answer is that the agglutinating complex of egg-water, fertilizin, and the jelly coat *in situ* have no free inhibitory groups. These are liberated, however, during the acid dissolution of the jelly coat (cf. 6). This would mean that the spermatozoa do not need any substance like octylalcohol to accomplish the breakdown of the jelly coat and fertilizin. There are some observations which support this view. For example, the agglutination of the sperm due to egg-water is often more spherical in type than that due to jelly solution, which tends to be more net-like, indicating that in this latter case additional groups exist which react with the tails (see also 5). Furthermore, the jelly coat is removed much more easily from newly fertilized than from unfertilized *P. lividus* eggs, which might depend either on an enzymatic breakdown by the sperm or on the dissolving action of the respiratory carbon dioxide (5, 6) and the acid which is released at the moment of fertilization.

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SILVER IMPREGNATION OF SPERMATOZOA[1]

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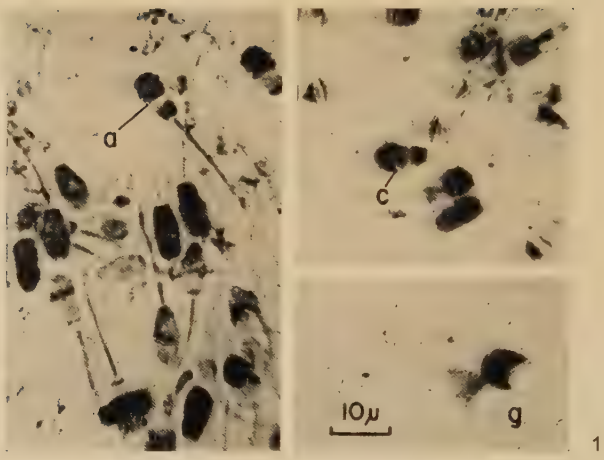
The recent admirable account in this Journal of ram sperm structure based on electron micrography (3) has further demonstrated the complex detail of these cells. Such morphological descriptions may be expected to serve significantly in the elucidation of the functional aspects of sperm biology. At present, however, theories concerning the physical mechanism for sperm activity are largely of a speculative nature (4).

While attempting to develop an autoradiograph technique applicable to spermatozoa previously exposed to radioactive phosphorous, impregnation of the spermatozoa by silver in the emulsion was suspected as the cause for numerous intensely stained cells adhering to the film (1). Further analysis proved the silver impregnation method to be an effective means of demonstrating simply and clearly considerable detail not found in preparations treated by the usual staining procedures. As some of these features supplement certain interpretations based on electron micrographic study of spermatozoa, a note is relevant at this time. The silver reactions may be regarded as primarily surface absorption phenomena; nevertheless the internal protoplasm of the cells frequently stains throughout. Furthermore, variations in impregnation results indicate functional differences in the activity of the spermatozoa.

Silver impregnations were obtained on seminal spermatozoa from the cock, ram, dog, bull, and man and on spermatozoa collected from the vas of the frog, turtle, rabbit, and guinea pig. Air-dried smears of spermatozoa in seminal fluid or buffer diluent were treated with aqueous silver nitrate, rinsed, and exposed to light to permit the reduction of silver in accordance with the classical impregnation technique long used in protozoological studies. Two to five per cent $AgNO_3$ gave good results, but higher concentrations resulted in excessive contamination by silver precipitation. Since the pH of the fluid in which the spermatozoa were suspended appeared to be a factor in the intensity of the silver reaction, a series of phosphate buffer diluents from pH 5.85 to 7.65 were tested in their effect on the impregnation. Best results were obtained between pH 6.5 and 7.5 corresponding more closely to the normal biological range and to the range of greatest motility of the spermatozoa.

In silver treated bull spermatozoa the head is clearly distinguished into an anterior portion, consisting of the acrosome which is surmounted by the galea capitis and

[1] This work has been done under grants administered by the Committee on Human Reproduction of the National Research Council.



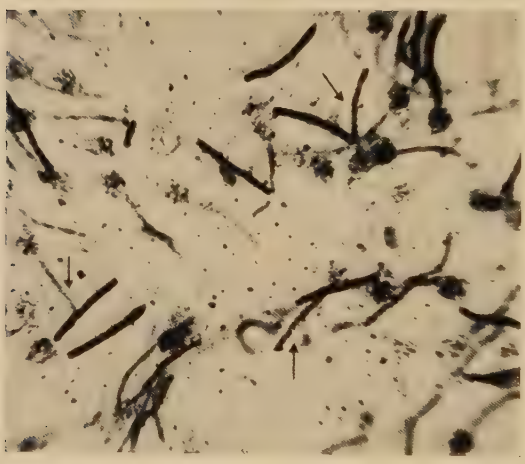


Fig. 1. Bull spermatozoa showing margins of the galea capitis (c) and of the acrosome (a). The galea capitis (g), removed by agitation of the spermatozoa, appears to have a furrowed or pitted surface (5 per cent $AgNO_3$).

Fig. 2. Middle-pieces of bull sperm treated with 5 per cent $AgNO_3$ showing various degrees of impregnation density. The helical structure, clearly seen in the original material, shows 20 to 24 coils per middlepiece.

which covers most of the nucleus, and a posterior portion, made up of the post-nuclear cap covering the remainder of the nucleus. The posterior limiting margin of the acrosome—coincidental with the equatorial "nuclear ring"—externally delineates the two parts of the head. The clear crescent-shaped unstained region, frequently but not always appearing beneath the anterior end of the galea capitis, may be an artefact due to both shrinkage of the nucleus and partial lifting of the

cap which occasionally is removed entirely from the head (Fig. 1). The posterior margin of the galea capitis is considerably anterior to that of the acrosome, leaving a region between generally known as the equatorial segment. The margin of the galea capitis subtends an angle of 130 to 140°. The gross structure of the bull sperm head and the boundaries of the galea capitis and of the acrosome are thus seen in silver impregnated cells to be similar to those of ram spermatozoa as demonstrated by the electron microscope. Little internal differentiation of the head is demonstrable beyond a tendency for a posterior-anterior argentophilic gradient.

The middle-piece of the bull spermatozoan is variable in its silver affinities. This structure, like the head, is quite susceptible to silver impregnation but shows an independent reactivity and may stain when the rest of the gamete is almost colorless, or may remain unstained when the head and the tail have marked argentophilic qualities. This variation depends on the condition of the spermatozoa at the time of treatment, but the pertinent factors in the middle-piece reaction are not clear. Neither ageing, dilution, or pH changes in the seminal fluid, nor concentration, exposure time, or pH changes in the $AgNO_3$ seem to play any consistently responsible role in the intensity of impregnation. The middle-piece of bull spermatozoa is spiraled beyond question (2), the major helix accomplishing 20 to 24 gyres along its length (Fig. 2). This is similar to the condition existing in ram spermatozoa, and in marked contrast to that of the non-spiraled human sperm cell.

The sperm tails frequently become densely impregnated, the entire sheath or involucrum staining. However, the technique is inadequate for demonstrating the helical nature of the tail indicated by more refined methods. The terminal fibrils can be distinguished, but usually are clumped into a single fine projection one to two micra in length.

Further work is in progress to ascertain the variations noted and to improve the definition and clarity of the procedure for its use as a rapid and effective stain for spermatozoa.

The writer is indebted to Everett Kosarick for taking the photomicrographs.

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THE CHEMICAL NATURE OF THE SO-CALLED KERATOHYALINE GRANULES OF THE STRATUM GRANULOSUM OF THE SKIN

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The chemical composition of the granules occurring in the cytoplasm of the cells of the stratum granulosum of the epidermis is unknown. It has been generally assumed that these granules are a result of the degeneration of the cells, the stratum granulosum being an "intermediate zone between life and death" (5). Since the granules stain in a similar fashion as does the chromatin; namely, with hematoxylin and other basic dyes, they were generally believed to be chromatin material derived from the breakdown of the nucleus.

In the following study evidence is presented which contradicts the concept that the keratohyaline granules are chromatin and which suggests an anabolic activity of the cells of the stratum granulosum.

Experimental Results and Observations: In order to test whether the granules contain chromatin material, they were examined for desoxyribosenucleic acid by means of the Feulgen reaction. The specificity of the Feulgen reaction for desoxyribosenucleic acid, if carried out under controlled conditions, has been established by numerous investigators (12, 3, 8, 10). In the epidermis of human and dog skin (fixed in Carnoy's acetic alcohol and 10 per cent formalin) the granules of the cells of the stratum granulosum were completely Feulgen-negative, while they stained strongly with hematoxylin. This finding agrees with observations made by T. Cornbleet (personal communication).

From these observations it can be concluded that the granules of the stratum granulosum do not contain desoxyribosenucleic acid. Since it seemed possible that in spite of the Feulgen-negative behavior of the granules there is a loss of desoxyribosenucleic acid from the nucleus of these cells similar to the loss of desoxyribosenucleic acid in pyknotic nuclei (6, 7), the amount of desoxyribosenucleic acid in nuclei of the stratum granulosum cells was compared with the amount of desoxyribosenucleic acid in nuclei of the malpighian cells. The desoxyribosenucleic acid was estimated by photometric measurements with an apparatus described by Pollister and Moses (9) and by the method of Schrader and Leuchtenberger (11). For the measurements of the desoxyribosenucleic acid in the nuclei, cells were selected in the stratum granulosum which were directly adjacent to the malpighian

layer and which showed in their cytoplasm, on hematoxylin stain, distinct granules. Cells lying directly beneath the keratin could not be measured since, due to their extreme spindle form, a computation of the volume was not possible.

In the following table the results are presented.

TABLE I

Amounts of Desoxyribosenucleic Acid (Feulgen) in Arbitrary Units in Nuclei of Epidermal Cells of the Dog

Type of Cells	Number Measured	Mean Nuclear Volume in Cubic Microns	Mean Amount of Desoxyribosenucleic Acid per Nucleus
Malpighian Layer	20	230 ± 26	2.3 ± 0.19
Stratum Granulosum	20	196 ± 21	2.0 ± 0.20

It is apparent that the desoxyribosenucleic acid content of the nuclei of the stratum granulosum is approximately the same as that of the malpighian layer and therefore no loss of desoxyribosenucleic acid seems to have occurred during the process of formation of the granules in the cytoplasm of the cells of the stratum granulosum.

For a further characterization, the granules were tested for ribosenucleic acid. The methods employed were the one of Brachet (1), using a combination of toluidine blue and ribonuclease, and the one of Flax and Pollister (4) using azure A. In both sets of experiments the stainability of the granules with toluidine blue and with azure A was completely abolished if the sections were treated with ribonuclease prior to the staining. It can therefore be concluded that the granules contain ribosenucleic acid. It should be pointed out, however, that the intensity of the stain in the granules with azure A and with toluidine blue is considerably less than with hematoxylin. Whether this observation is an indication that the ribosenucleic acid constitutes only a small part of the granular material and the stronger staining with hematoxylin is due to its unspecific staining of other acidic components of the granules, or whether the weaker staining with azure A and toluidine blue is due to the fact that these dyes stain only a part of the ribosenucleic acid (4, 11) cannot be decided at the present time.

Discussion of the Result. The Feulgen-negative behavior of the granules in the cytoplasm of the cells of the stratum granulosum is against the view that the granules are chromatin and therefore a breakdown product of the nuclear material. The unchanged amount of the desoxyribosenucleic acid in the nuclei of these cells and the presence of ribosenucleic acid in the cytoplasmic granules are both inconsistent with the general concept that these cells are degenerating cells.

Accepting the above evidence, it may still be maintained that we deal here with a very early phase of degeneration. However, under pathologic conditions where all stages of degeneration have been observed in the epidermis and other tissues, there does not seem to be an equivalent picture. If chromatin is lost from the nucleus and appears in the cytoplasm, it is Feulgen-positive, and moreover the desoxyribose-

nucleic acid in such nuclei is considerably less than in nuclei of nondegenerating cells (6, 7). The stratum granulosum cells, furthermore, lack transitional stages so commonly observed in degeneration and necrosis. Pyknosis, which occurs occasionally in the uppermost layer of the stratum granulosum, is an abrupt change and appears to be unrelated to the presence or absence of granules.

The presence of ribosenucleic acid in the granules of the cells of the stratum granulosum rather suggests that such cells might be engaged in the synthesis of substances related to the process of keratinization for it has been shown in a wide variety of cells that such a chemical condition in the cytoplasm characterizes cells where protein synthesis is taking place (2). This concept seems to be supported by the fact that Caspersson *et al* (2) by the method of ultraviolet absorption found an accumulation of ribosenucleotides during the process of keratinization (2).

Cytochemical studies of intracellular nucleoproteins during disturbances of the normal process of keratinization (parakeratosis and hyperkeratosis) are now in progress.

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